Sensing Pheromones and Host Volatiles in Moths and Flies: From Molecules to Detection to Odor-guided Behavior

Dissertation

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INTRODUCTION

Chemical communication

'The code was first broken […] after extraction of a half million female silkworm pheromone glands and 30 years of classical chemical analyses. [...] This work showed that there was nothing magical about the communication system, and chemists around the world were "attracted" to this area of research on insect pheromones'

Roelofs, 1995, Colloquium Paper, National Academy of Sciences

Such groundbreaking evidences do not provide much space for the idea of magic in the world of chemical ecology. Nevertheless, the imagination that animals can be guided over long distances of several kilometers by a few invisible volatile molecules is difficult to grasp and has still something magical. Odors present themselves in an enormous variety of molecular structures, concentrations and ratios, consequently, they cannot be easily defined in single parameters, fashioning the sense of smell with great complexity. In order to communicate, organisms use semiochemicals[1]. First reports on chemical communication can be traced back at least to the 16th century. The French author François Rabelais vividly described how the vulval tissue of a female canine, which is lubricated on a woman, attracts many males:

'But the best was at the procession, in which were seen above six hundred thousand and fourteen dogs about her, which did very much trouble and molest her, and whithersoever she passed, those dogs that came afresh, tracing her footsteps, followed her at the heels, and pissed in the way where her gown had touched.' [2]

These semiochemicals not only have such a forceful influence on canines. Other organisms ranging from bacteria, plants, invertebrates and vertebrates, use chemical communication for essential life tasks. Invertebrates like the sea hare *Aplysia,* a major model in neurobiology, use pheromones for attraction (Painter et al.1998). The urine of rodents mainly provides olfactory cues inducing sexual behavior, aggression or attraction (Johnston2003). Plants use volatile chemicals as defense strategy (Dicke2009, Kessler and Baldwin2002) and bacteria benefit from chemical cues while searching nutritious food sources (Adler1975).

[1] Chemicals, which are released by a sender, provide information for a receiver and affect his behavior =(Law and Regnier1971)=(Law and Regnier1971).

[2] François Rabelais, 1565, Gargantua and his son Pantagruel, book 2, chapter 22, english translation, Sir T. Urquhart of Cromarty, Moray press, 1894

Pheromone communication in insects

This thesis focuses mainly on special semiochemicals that are used by distinct species for intraspecific communication. Those so-called pheromones are diverse in their function. Depending on the purpose, insects use pheromones for many tasks, e.g., as alarm pheromone in aphids (Bowers et al.1972, Kislow and Edwards1972, Vandermoten et al.2012) or bees (Bortolotti and Costa2014) in order to warn conspecifics against enemies. Bees also produce pheromones for colony defense (Boch et al.1962). Pheromones may also act as aggregation pheromone, for example, that are produced by the boll weevils to indicate feeding sites (Tumlinson et al.1969). A major group of pheromones, the sex pheromones, are used by insects in order to find a mate. Typically, a male will be attracted by a conspecific female-released pheromone. The sex pheromone system of butterflies and moths (lepidoptera) is one of the most extensively investigated (Cardé and Minks1997). The first experimental investigations on moth' sex attractants at the end of the 19th - beginning of the 20th century constituted important milestones in the history of chemical communication (Fabre1879, Forel1910). Fifty years later the first sexual attractant, bombykol, was identified in the silkmoth *Bombyx mori* (Butenandt1959). Karlson and Lüscher1959) first used the term pheromone. After the discovery of the first sex attractant interest in identification of pheromone compounds grew. Some species, like the leaf-mining moth *Cameraria ohridella* (Svatoš et al.1999) or *Bombyx mori* (bombykol, Butenandt1959), have single active pheromone compounds causing male attraction, while other species have a species-specific blend with a defined ratio of its components - most probably in order to prevent hybridization, i.e. mating with closely related species (e.g. Groot et al.2006, Vickers et al.1991). Females of the tobacco budworm *Heliothis virescens* produce a blend consisting of up to seven compounds (Pope et al.1982, Roelofs et al.1974, Tumlinson et al.1975, Vetter and Baker1983). Notably, only little variation in the ratios of the individual components causes a loss of attraction of conspecifics males (Klun et al.1979, Ramaswamy and Roush1986, Vickers et al.1991). Thus, the relative amount of the pheromone components within a blend is important for the specificity of the blend. In several lepidopteran species, males carry scent brushes and emit a male-specific pheromone (Baker et al.1981, Birch and Poppy1990, Cardé and Minks1997, Pliske and Eisner1969). Such odors are reported to be involved in courtship and act as close range pheromones. The abdomen of males of, e.g., *H. virescens* carries scent brushes, called hair pencils, which release volatiles enhancing mate acceptance in conspecific females (Hillier and Vickers2004, Teal and Tumlinson1989). In the vinegar fly *Drosophila melanogaster*, an increase in the female's receptivity is mediated by the male-specific pheromone 11-cis-vaccenyl acetate (cVA, Bartelt et al.1985, Ha and Smith2006, Kurtovic et al.2007). But in contrast to the pheromones described above, cVA alone mediates several behaviors dependent on the social context (Ejima2015 and citations therein) as shown in Figure ***1***. It serves as an aphrodisiac during courtship by enhancing the female's receptivity, and as an aggregation pheromone for both sexes in long range. At the same time it can cause aggression in males in close range. Furthermore, since cVA is transferred to the female abdomen during mating, it prevents males from copulating with mated females acting as an anti-aphrodisiac.

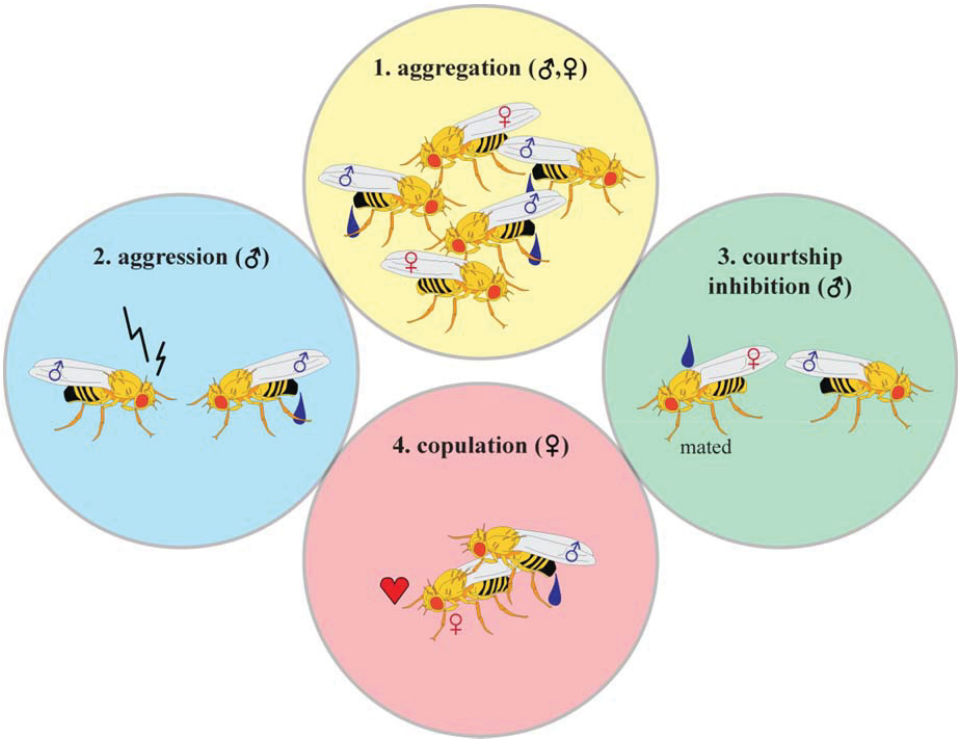

Figure 1: A multifunctional pheromone in *Drosophila melanogaster*.
Depending on the social context the function of cVA as an aggregation, sex or aggression pheromone. Based on Ejima (2015). Illustration of *Drosophila*: M. Stensmyr.

The insect olfactory system

Insects are adapted to their habitat and as a consequence morphologies seem to be evolved according to their needs (Darwin1859). In line with this, not only the habitat but also chemical communication such as the pheromones system might affect insect antennae morphologically, since they vary enormously in size and shape as shown for three species in figure 2. However, irrespective of size and shape of the olfactory organs the basic principles of the olfactory system are considered to be similar in insects. The organization of the olfactory system is shown in Figure 3 using the example of a moth. Insects detect environmental odors via olfactory hair-like structures, called sensilla, mainly located on their antennae. The antenna possesses three major types of sensilla, sensing different types of chemical classes (Shields and Hildebrand2001, Silbering et al.2011): sensilla trichoidea, sensilla basiconica and sensilla coeloconica. Basiconic sensilla are shown to detect food and/or plant-related compounds like terpenes, while coeloconic sensilla mainly detect amines and acids as reported in *D. melanogaster* (Abuin et al.2011, Benton et al.2009, Silbering et al.2011). Moreover, in lepidopteran species such as *B. mori* it is observed, that coeloconic sensilla also detect plant volatiles (Pophof1997). Irrespective of the insect species, pheromones are perceived by the third type, the trichoid sensilla. In female moths,

this sensillum type often detects plant volatiles beside pheromones (Hillier et al.2006, Shields and Hildebrand2000).

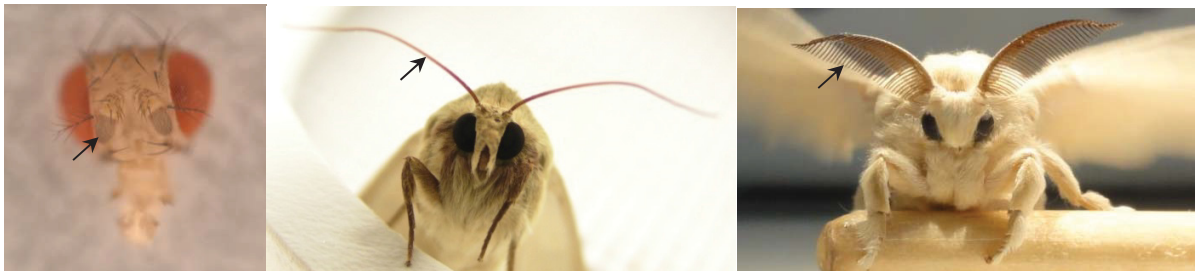

Figure 2: Differences in shape and size of insect antennae (black arrow).
Female head of *Drosophila melanogaster* (left), *Heliothis virescens* male (middle) and *Bombyx mori* male (right). Photo: left, V. Grabe, middle and right, E. Schuh

The detection of odor molecules is mediated by olfactory sensory neurons (OSNs), which are housed in the sensilla (Leal2003, Prestwich1993). The dendrites of the OSNs are bathed in sensillum lymph, a barrier between the environment and the OSNs. In adult moths and flies, each sensillum houses up to four OSNs, whereas in caterpillars the amount of OSNs per sensillum can be higher. The caterpillar of *H. virescens* contains up to 19 neurons in their olfactory sensilla (Laue2000) and 21 OSNs were observed in the olfactory organ of *Drosophila* larvae, the dorsal organ (Fishilevich et al.2005). Each OSN in adults expresses primarily one chemosensory receptor being involved in olfaction. Two types of these chemosensory receptors are expressed on the insect antenna: OSNs that are housed in coeloconic sensilla mostly express ionotropic receptors (IRs, Benton et al.2009), while olfactory receptors (ORs) are expressed in the neurites of basiconic and trichoid sensilla (Clyne et al.1999).

In insects, ORs function in combination with the co-receptor Orco as a heterodimer (Larsson et al.2004, Neuhaus et al.2005, Vosshall and Hansson2011). Odorant molecules bind to the OR subunit, while Orco forms an ion channel and is involved in the dendritic localization of the ORs (Larsson et al.2004). Odorant binding leads to an ion flux whereby a chemical signal is transformed into an electrical one. The resulting action potentials can be measured and visualized using single sensillum recordings (SSR; see also methodology), whereby revealing distinct response characteristics like spontaneous activity, response dynamic and excitation or inhibition of the OSNs. These response characteristics depend on the expressed chemosensory receptor (Hallem et al.2004).

Pores on the sensillum wall enable odor molecules to enter the cuticle. Odor molecules in the sensillum lymph bind to odorant binding proteins (OPBs) (Vogt1987); in trichoid sensilla of lepidopteran to pheromone binding proteins (PBPs) (Krieger et al.1993, Vogt et al.1991). The

mechanism by how OBPs and PBPs are involved in odor detection and recognition is still unclear. It is considered that OBPs bind odorant molecules, aiding a hydrophobic odorant molecule to reach the membrane of the OSNs (Leal2003, Prestwich1993).

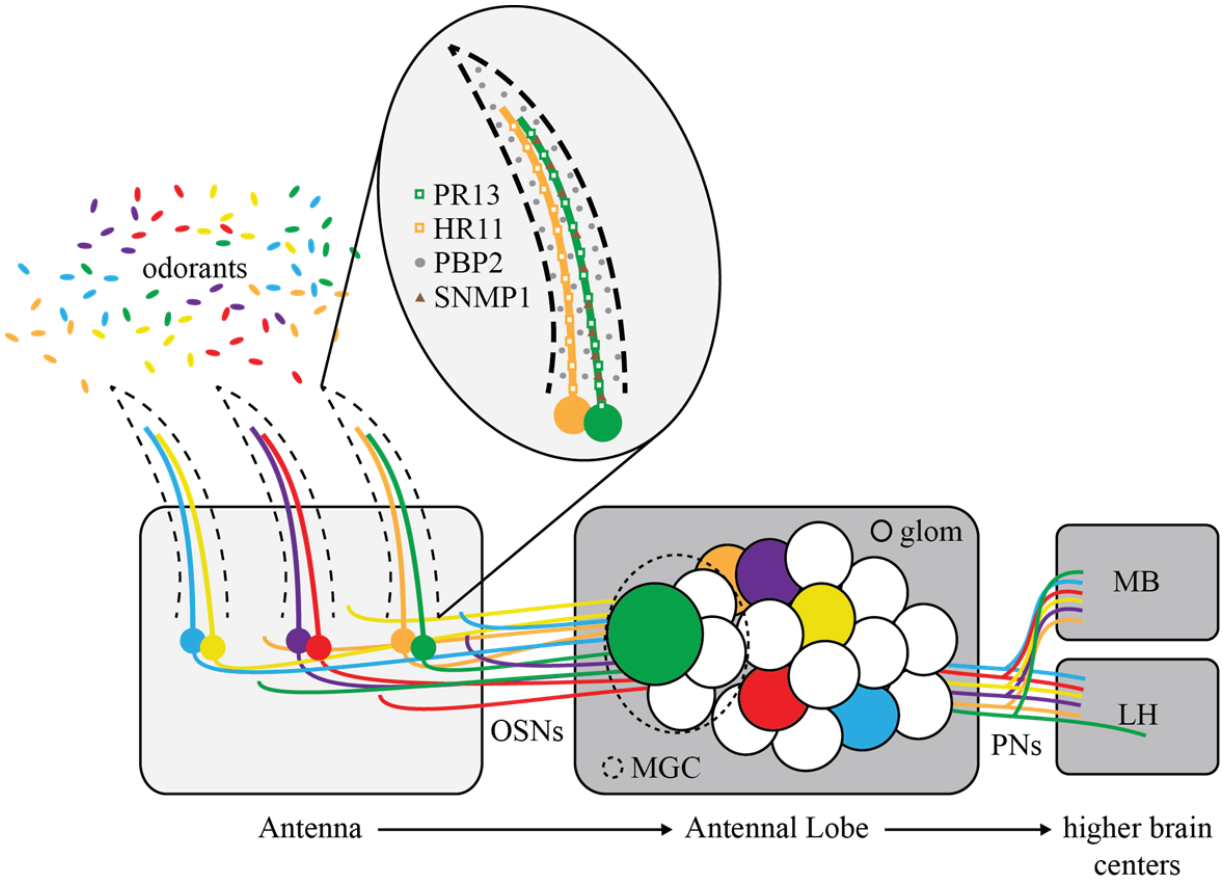

Figure 3: Principle organization of the olfactory system in the moths *H. virescens*.
Sensilla at the sensory level (light grey) including a magnification of a long trichoid sensillum detecting the major sex pheromone component of *H. virescens*. Processing level (dark grey): (glom) glomerulus, (MGC) magroglomerular complex, (PNs) projection neurons, (LH) lateral horn, (MB) mushroom body. Based on investigations made in male *H. virescens* (Baker2009, Berg et al.2014, Große-Wilde et al.2007, Krieger et al.1993, Pregitzer et al.2014).

In pheromone-responsive sensilla additional molecular elements are involved in pheromone recognition, namely sensory neuron membrane proteins (SNMPs), that are expressed in the dendritic membrane (Benton et al.2007, Rogers et al.2001, Rogers et al.1997). It is considered that SNMPs support the release of odor molecules from the PBP/odorant complex to the receptor (Benton et al.2007). In general, SNMPs contribute to the high sensitivity of pheromone detection in insects, which, is demonstrated in *H. virescens* (Pregitzer et al.2014) or in *D. melanogaster*

(Benton et al.2007, Jin et al.2008). The experiments by Benton et al. (2007) show, that flies, which lack SNMP1 in its OR67d-expressing trichoid sensilla (at1), loose its sensitivity to cVA. Hence, it is believed that additional factors to the ORs play a role in the recognition of pheromones and might enable highly sensitive pheromone detection.

OSNs expressing the same ORs converge in the same neuropil structure, glomerulus (Koontz and Schneider1987), found in the first processing center of the olfactory brain, the antennal lobe (AL) (Gao et al.2000, Vosshall2000).Within the AL specific local interneurons (LNs) are responsible for the modulation of intra- and interglomerular olfactory information (Matsumoto and Hildebrand1981, Seki and Kanzaki2008). Subsequently, projection neurons (PNs) transfer the information to higher brain centers, namely the lateral horn or the mushroom body.

Pheromone detection in insect larvae

In noctuid moths, single pheromone components are detected by highly specific ORs, so-called pheromone receptors (PRs), which represent a subgroup of ORs (Große-Wilde et al.2007, Krieger et al.2005, Krieger et al.2004, Sakurai et al.2004, Wang et al.2011). As an example, HR13 is the PR expressed in 80 % of the trichoid sensilla of male *H. virescens*. Only the major sex pheromone component released by conspecific females activates the HR13-expressing neurons (Krieger et al.2004, Kurtovic et al.2007). Surprisingly, chemical communication via pheromones seems to be important not only for adults, but also for larvae. Some lepidopteran species demonstrate the capability to detect female-specific pheromones in the larval stage (Jin et al.2015, Poivet et al.2012, Tanaka et al.2009, Zhu et al.2016). Whereas in *Bombyx mori,* larvae are not affected by the pheromone bombykol at the behavioral level (Tanaka et al.2009), caterpillars of the noctuid moth *Spodoptera littoralis* prefer food sources containing the female sex pheromone regardless of stage and sex (Poivet et al.2012). Poivet et al.2012) showed in addition that the adult PRs are not expressed in larval antennal tissue, but that the expressed PBPs might be involved in larval sex pheromone detection. Such findings are supported by studies in larvae of *B. mori* (Tanaka et al.2009). It is discussed that the highly sensitive pheromone detection system in male moths is provided not only by the specific PR, but is the result of the combination of the three molecular elements, PBP, SNMP and PR. The molecular elements involved in pheromone detection of caterpillars were not often in the focus of previous research. Consequently, it is also not known, if similar elements (PR, PBP, SNMP) are involved in the sensitivity of pheromone detection in larvae similar to adult males. In chapter 1 we address, whether the larvae of the budworm *H. virescens* detect female-specific pheromone components, and whether caterpillars and adult males use the same molecular elements for pheromone detection.

The pheromone system and sexual dimorphism

Due to their different ecology males and females have to deal with different tasks that is reflected in sexual dimorphisms on different levels of the olfactory pathway (Grabe et al.2016, Hansson and Anton2000, Hansson et al.1992, Koontz and Schneider1987). Females have to find suitable oviposition sites to ensure the presence of sufficient and appropriate food for their larvae. Consequently, sensing host-plants becomes even more crucial after mating. The task of a male is to find a conspecific female. Males have to be highly sensitive to pheromones, since they are attracted to the female-released pheromone even over very long distances. Thus, males express the three molecular elements, PBP, SNMP and PR. Certain elements like PBPs and/or SNMP1were also identified in female antennae (Krieger et al.1996, Rogers et al.2001, Zielonka et al.2016). Nevertheless, the expression in females is often lower than in males and especially the set of PRs in females is only present to some extent compared to males (Krieger et al.2004, Steinbrecht et al.1995, Zielonka et al.2016). Females either lack pheromone-responsive sensilla completely (*Antharea sp*.: (Steinbrecht et al.1995), or the expressed ORs within trichoid sensilla are different between the sexes (*B. mori*: Krieger et al.2005), or they possess only a subset of the PRs expressed in conspecific males, like in *H. virescens* (Almaas and Mustaparta1991, Hillier et al.2006, Krieger et al.2004, Zielonka et al.2016).

Sexual dimorphism on the antenna is also represented in the AL. Based on functional representation the olfactory system in lepidopteran males is divided into two different subsystems that comprises the following regions in the AL (Figure 3): the magroglomerular complex (MGC, Bretschneider1924) for pheromone processing and the ordinary glomeruli for the processing of general, non-pheromone odorants (Anton and Hansson1999, Hansson and Anton2000, Hansson et al.1989, Mustaparta1984)**.** The MGC consists of at least two or more glomeruli like in *B. mori* (Koontz and Schneider1987) and in *H. virescens* (Hansson et al.1995). All OSNs of pheromone-responsive sensilla send their axons to the MGC (Baker2009). In contrast to that, females do not have such a structure at the entrance of the AL. Instead they possess two large female glomeruli (LFG) that encode conspecific pheromone components as well as plant volatiles (Berg et al.2002, Hillier et al.2006, Shields and Hildebrand2001). Both subsystems can influence each other (see section Interaction with pheromones). In contrast, sexual dimorphism in the vinegar fly olfaction is not as distinct as in lepidopterans (Grabe et al.2016, Vosshall2008). A higher amount of trichoid sensilla and a larger cVA-responsive glomerulus DA1 distinguish males from females (Grabe et al.2016).

Pheromone and host plant detection in female moths

Adult females use pheromones in order to choose a mating partner (Hillier and Vickers2004). After selecting an appropriate sex partner, a female moth has to search for a suitable oviposition site; the latter is mediated by host plant volatiles. Such volatile chemicals do not only mediate

the attraction towards a preferred plant, it can also induce egg laying in female insects (Allmann et al.2013, Bisch-Knaden et al.2018, De Moraes et al.2001, Spaethe et al.2013b, Tingle et al.1990). Besides basiconic sensilla, trichoid sensilla of female moths detect plant volatiles as well (Hillier et al.2006, Shields and Hildebrand2000), suggesting an important role of plant volatiles for female moths.

Mate acceptance of a female is mediated by male-specific pheromones. From studies on *H. virescens* it is known, that a female detects male-released hair pencil compounds by her trichoid sensilla (Hillier et al.2006). Moreover, not only plant volatiles and male-specific pheromones are detected by female trichoid sensilla (Hillier et al.2006), but also the pheromone of conspecific females (so-called autodetection) as described for *H. virescens* (Hillier et al.2006) or the leafworm *S. littoralis* (Ljungberg et al.1993). The role of autodetection is still under debate. Presentation of the female-specific pheromone to a female chestnut moth induces calling behavior in the same female at a distinct distance from other calling conspecifics (Den Otter et al.1996). Thus, it is considered that autodetection facilitates synchronous calling, but simultaneously leads to avoidance of other calling females. In other species, like *B. mori*, females are considered to be anosmic for their own pheromone bombykol (Schneider1957). It is conceivable that females of *B. mori* do not detect themselves or conspecific females, but they might detect hair pencil compounds instead. Although hair pencil-like structures are exclusively observed in the conspecific males, male-specific pheromones are not yet identified (Anderson et al.2009). Similar to males, female silkmoths possess a high number of long-sized trichoid sensilla (Heath et al.1992, Shields and Hildebrand1999, Steinbrecht1970), although female antennae of moths often lack this sensillum type (Scheffler1975). Several studies examined physiological aspects of long-sized trichoid sensilla in females like the dynamic of odor responses (Heinbockel and Kaissling1996), but a large odor screening is not implemented to understand the quality or quantity of volatiles needed to activate the OSNs of this sensillum type (receptive range). Investigations from Priesner (1979) suggest that OSNs of long trichoid sensilla in females are broader tuned than those of males. Long trichoid sensilla in males and females seem to be completely different with regard to their function. In female silkmoths this sensillum type detects acids and terpenes like the known plant-emitted compound linalool instead of detecting bombykol and bombykal (Heinbockel and Kaissling1996, Priesner1979). Only a single behavioral study shows that females prefer to oviposit in the presence of the terpenes valencene and α-humulene (Damodaram et al.2014). But here they did not test volatiles that activate long-sized trichoid sensilla like linalool. Furthermore, besides long trichoid sensilla female possess also medium-sized trichoid sensilla (Steinbrecht1970). Neither the functional properties of medium trichoid sensilla nor their biological role have yet been investigated in female *B. mori*. Males that are highly attracted by the female-specific pheromone bombykol respond with an intensive wing fanning behavior (Butenandt1959), whereas females are considered to be almost stationary throughout their life. In fact, the ecological significance of trichoid sensilla in female *B. mori* is still not clearly understood. In chapter 2 of my thesis I characterized the receptive range of OSNs that are housed in long trichoid sensilla of female *B. mori,* considering that

trichoid sensilla of female moths serve as detectors for both pheromone and plant volatiles. In addition, I characterized medium trichoid sensilla, since they are so far not investigated in females. In order to study the biological relevance of the physiological active volatiles I subsequently tested such compounds at the behavioral level.

Interactions with pheromones

In their natural habitat insects never perceive pheromones in an odorless background. The environment is rich of volatiles being released by a huge diversity of plants. Only a small portion of such chemicals do have a value for the insect. Thus, dependent on the context insects are confronted with the task to filter out the meaningful odors in order to identify food and host plants as well as mating partners, conspecifics or predators (Bruce et al.2005, Visser1986). Additionally, the ecological relevance of an odor can vary for the different life stages of an insect. Some species like *B. mori* do not feed at all throughout their adult life stage, but throughout their larval stage they mainly feed on leaves of the mulberry plants (Ishikawa et al.1969). Other species rely on food during all life stages, like the generalist *Heliothis virescens*, whose larvae feed on leaves while the adults feed on nectar (Cunningham and Zalucki2014, Fitt1989). Volatiles of such food sources are detected by the insect and serve as olfactory cues to locate the food source. Such cues provide information that, for example, leads to attraction toward a host plant (Bisch-Knaden et al.2018, Dweck et al.2013). *Heliothis virescens*, for example, is a pest species on several plants, like cotton, tobacco, soybean or tomato (Cunningham and Zalucki2014, Fitt1989), and the insect detects plant-related volatiles like linalool, (Z)3-hexen-1-ol or β-caryophyllene (Rostelien et al.2005, Skiri et al.2004, Stranden et al.2003). Wind tunnel experiments demonstrate that both male and female moths are attracted to host odors (De Moraes et al.2001, Tingle and Mitchell1992, Tingle et al.1990).

Do pheromone and environmental odors like plant volatiles interfere with each other? Several studies addressed the interaction of plant odors and pheromones; however, there are currently contradictory findings in this field of research. On the one hand, in several species including *Spodoptera exigua, Cydia pomonella*, *Helicoverpa ze*a and *Bombxy mori*, the neuronal and behavioral responses to the female-specific pheromone is increases in males when adding plant volatiles (reviewed in Deisig et al.2014, Gurba and Guerin2016). On the other hand, simultaneous presentation of plant volatiles can also inhibit pheromone detection as demonstrated in species like *Spodoptera littoralis*, *Bombxy mori*, *Agrotis ipsilon* (Chaffiol et al.2012, Deisig et al.2012, Hatano et al.2015, Kaissling et al.1989, Party et al.2009). Another interesting example of plant-pheromone interaction was studied in more detail in *Heliothis virescens*. Here, scientists examined the influence of plant-related volatiles on the peripheral pheromone response and the involved molecular elements (Hillier and Vickers2011, Pregitzer et al.2012). In short, plant volatiles reduce pheromone responses when simultaneously presented with either the major (Z11-16:Ald) or an important minor (Z9-14:Ald) sex pheromone

component of *H. virescens* (Hillier and Vickers2011). Pregitzer and co-workers (2012) demonstrate that the pheromone-plant interaction for Z11-16:Ald occurs at the level of the receptor. Nevertheless, the behavioral consequence of this interaction is not yet known. In chapter 3 of this thesis I therefore investigated pheromone-guided behavior in the presence and absence of plant volatiles by using a background consisting of either single synthetic plant volatiles or of the headspace of a host plant.

The vinegar fly *D. melanogaster* lives, mates and oviposit on rotten fruits and feeds on the growing yeast (Hansson and Stensmyr2011). During courtship the male-specific pheromone cVA is involved, that enhances the females' receptivity during courtship (Ejima2015). How does the constant exposure to food odors have an effect on the perception of cVA in female flies? To my knowledge the only investigations on pheromone interaction with a general odorant in females are demonstrated in the beetle *Rhynchophorus palmarum* and in *Drosophila melanogaster*. In the first example sugarcane enhances the female attraction toward the male-released aggregation pheromone (Oehlschlager et al.1993, Said et al.2005). In the second example cVA affects the attraction towards vinegar specifically in female *D. melanogaster*, although cVA and vinegar can be detected by both sexes (Lebreton et al.2015). Since the interaction of pheromones with other odorants seems to be complex, the fourth chapter of my thesis focuses on the pheromone-food interaction in female *D. melanogaster*. Moreover, in this chapter we investigated in particular the so far unknown underlying neuronal mechanisms being involved in such a chemical interaction. Does the interaction already take place on the antenna at the receptor level (Or67d, Kurtovic et al.2007)?

Methodology

In order to examine chemical communication in insects at different levels of the olfactory pathway, I used a variety of methods. I therefore mainly compared the neuronal level in the periphery and the behavioral consequence. When testing the odor response of insects, odor stimuli are important, since many studies use only single, synthetic odorants. A single ecological significant component of an odor can already induce a behavior regardless of the other compounds of the natural blend (reviewed in Haverkamp et al.2018), but primarily, a combination of several odorants of a natural blend is important to provide meaningful olfactory information for the insect (Riffell et al.2009, Spaethe et al.2013a). The second methodical consideration concerns the concentration, as many studies use unnaturally high concentrations of odorants, although insects are confronted with relatively low doses in nature. In order to mimic more natural situations, I used natural odor blends and applied them to the insects in ecologically relevant concentrations. To do so, I used the advantage of odor collections by collecting headspaces of whole plants) and analyzed the odor collections with gas chromatography coupled to mass spectrometry (GC-MS) (chapter 2 and 3). Hereby, I could identify single plant-emitted volatiles depending on the physiological state of the plant. Furthermore, I was able to separate a

natural odor blend into its single compounds and hence identify the ecologically relevant chemicals.

Throughout my thesis, I examined the physiological activity of such relevant olfactory cues using single sensillum recording (SSR). This technique enabled me to examine the receptive range of single OSNs in trichoid sensilla (chapter 1, 2 and 4). Depending on the nature of the sensillum wall, SSR can be performed in two ways: First, sensilla are cut manually and a glass capillary filled with ringer solution is placed over the tip (chapter 2, cut tip method: (Kaissling1974). Second, a tungsten electrode is directly poked into the sensillum (chapter 1 and 4). Using these two approaches, I could investigate different stages of development and several insect species: starting in caterpillars up to the adults, from lepidopteran to flies. By doing so, I addressed the questions, if the olfactory sensilla of larvae detect adult female-specific pheromone compounds (chapter 1) and furthermore, which chemicals activate OSNs of trichoid sensilla of adult females (chapter 2). In chapter 4, I investigated the food-pheromone interaction in the periphery of the vinegar fly using SSR in order to study a putative interaction on the antenna. Moreover, beside the short generation times and the simplicity in rearing, *D. melanogaster* used in chapter 4, is a genetic toolbox making it a perfect model organism for specific manipulations in order to narrow the synergistic effect down to a specific level.

After knowing the detection capability of insects, the next questions arose: Which ecological relevance do detectable odors have? In chapter 3 I therefore investigated the behavioral consequences of pheromone-plant interaction in *H. virescens* (Hillier and Vickers2011, Pregitzer et al.2012), since plant volatiles suppress neuronal responses towards the major and minor pheromones component at the sensory level (Hiller and Vickers, 2011; Pregitzer et al., 2012). Moreover, I wanted to compare the behavioral performance to the pheromone in addition to either single plant-related volatiles - as being used in previous studies - or the headspaces of whole plants. For observing odor-guided flight behavior in insects in the laboratory, I used a 60-year old behavioral assay, the wind tunnel (Figure 4A). Combining the wind tunnel assay with modern computer technology enables the tracking of flying moths in three dimensions and with a high resolution while responding to an odor plume. As a result, odor-guided flight behavior, in particular searching and navigational strategies can be investigated in more detail. Searching behavior to an attractive odor stimulus is described in many species like canine (Jezierski et al.2016), or ants (e.g. Buehlmann et al.2014), as well as in lepidopteran (Cardé1996, Cardé and Minks1997, Kennedy1983, Vickers and Baker1997). In sum, if a flying insect encounters an attractive odor plume, which varies enormously depending on odor source, wind speed, wind direction and environmental structures (Murlis1992), it aligns upwind and navigates towards the odor source, which is, for example, a pheromone-releasing female. Losing the filamentous plume triggers a characteristic search behavior (casting and zig-zagging) that enhances the probability to find the plume again (Cardé and Minks1997, Hardie et al.2001, Vickers and Baker1994). Using this knowledge in combination with new tracking software, in chapter 3 I analyzed

parameters of such search strategies in the wind tunnel to study insect interaction with pheromone and plant volatiles in more detail.

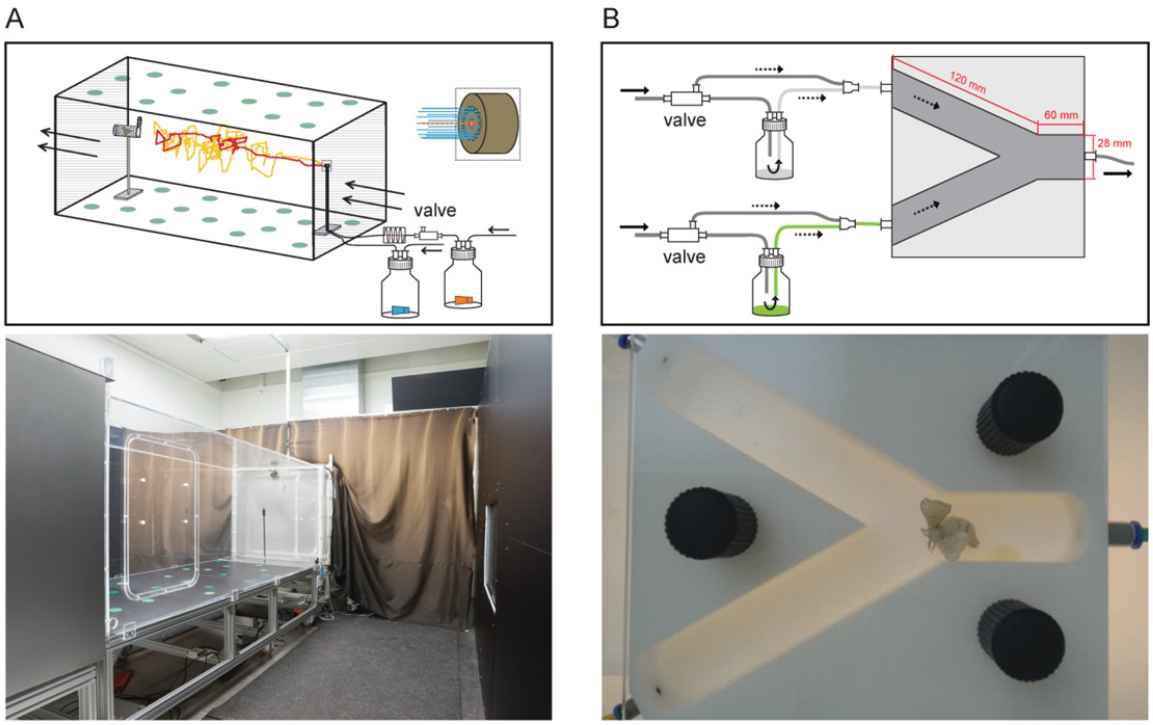

Figure 4: Behavioral assays to investigate odor-driven behavior in flying and walking insects.
(A) Upper panel represents a scheme of a wind tunnel assay with two flight patterns of a male moth (red and yellow) being attracted by the female pheromone. Odorant-containing bottles are connected with the outlet nozzle (A') and hence, volatiles are transferred into the wind tunnel. Arrows indicate the air flow. Lower panel represents a photo of the wind tunnel. **(B)** A scheme of the Y-maze assay including the stimulus presentation is shown in the upper panel. Each odor-containing bottle is connected to a valve, which pulses the volatiles into the two arms of the Y-maze. Arrows indicate the air flow. The lower panel shows a photo of a female silkmoth in the Y-maze. Photo: lower left, Anna Schroll, lower right, E. Schuh

Walking insects follow similar navigational strategies to find an odor source (Bell and Tobin1981, Willis2005). Due to domestication *B. mori* lost its ability to fly. Therefore, males walk upwind when entering a female-specific pheromone plume and start to counterturn in order to follow the plume (Butenandt et al.1961). On the contrary, conspecific female silkmoths have not been shown to navigate towards an odor source. For analyzing the hedonic valence of an odor, the Y-maze is a simple behavioral assay for walking insects (Baker and Cardé1984, Dethier1947). Within a Y-maze the insect can choose either between two different odors or between an odor and the control. In chapter 2 I established and modified the Y-maze for female

B. mori (Figure 4B) to create a bio assay for the silkmoths that allowed detailed analyses of the walking behavior. I connected the two-choice assay to an air flow and a camera. Thus, behaviors like up- and downwind movement, oviposition and wing fanning could be monitored and, consequently, I could demonstrate that female silkmoths show odor-guided behavior.

Aim of this thesis

With this thesis, I want to further 'break the code' of chemical communication down to the sensory and the behavioral level in order to discover the 'magic' of pheromones and host volatiles. In other words I want to investigate the role of pheromones and host volatiles for moth and flies at two major levels of the olfactory system in order to understand odor detection and its behavioral consequence.

First, I was involved in a study highlighting the importance of pheromones in the larval system. Using single sensillum recording we demonstrate that larvae of the budworm *Heliothis virescens* detect female-specific sex pheromone compounds. The ability to detect pheromones is probably based on the expression of the same set of molecular elements (HR6 and HR13, PBP1 and PBP2, SNMP1) as also conspecific adult males use in order to detect pheromone components (chapter 1).

Second, I demonstrate the significance of pheromone detection structures in female moths. Trichoid sensilla in female moths are considered to detect pheromone and plant volatiles (Shields and Hildebrand2000). However, female silkmoths (*Bombyx mori*) are not known for their ability to detect pheromone compounds, although they possess a large number of trichoid sensilla. A role of trichoid sensilla in host recognition is suggested, but the significance of such pheromone detection structures in *B. mori* is still poorly investigated. Therefore, in chapter 2 of this thesis I underline the importance of trichoid sensilla in host recognition in female silkmoths by characterizing two types of trichoid sensilla, long- and medium-sized, which are mainly tuned to host plant volatiles. This is strengthened by the result that mating influences the sensitivity to plant volatiles drastically and that females show different behaviors, like attraction and oviposition, after sensing plant-related odorants.

Third, I investigated the impact of natural plant odor sources on sex pheromone attraction in male moths using the wind tunnel and GC-MS analysis. The behavioral significance of inhibitory effects in plant-pheromone interaction is mainly examined at the neuronal level, but poorly understood at the behavioral level. My results demonstrated that the headspace of plants do not influence pheromone-guided flight behavior of male *H. virescens*, whereas unnaturally high doses of certain synthetic plant odorants decrease pheromone attraction (chapter 3).

Fourth, in my last chapter, I was involved in a study investigating the neuronal mechanism of food-plant interactions in *Drosophila melanogaster*. Contrary to *H. virescens*, in female vinegar

flies this interaction does not take place at the periphery, but at the level of the AL. We were able to show that a food odor, namely vinegar, enhances the sensitivity to the sex pheromone cVA that is mediated by electrical synapses between excitatory LNs and PNs (chapter 4). As a behavioral consequence, vinegar enhances the females' receptivity during courtship.

OVERVIEW OF THE CHAPTERS

Chapter 1

Larval sensilla of the moth Heliothis virescens respond to sex pheromone components

In this chapter I investigated the larval pheromone system using single sensillum recordings (SSR) and fluorescence *in situ* hybridization. Our data demonstrate that the larval large basiconic sensilla B2 of *H. virescens* detect the two major female-specific sex pheromone components, Z11-16:Ald and Z9-14:Ald. Furthermore, we could demonstrate that both male and female larvae express the pheromone receptors HR13 and HR6, as well as the sensory neuron membrane protein SNMP1 within olfactory sensory neurons (OSNs) of these sensilla. In addition, the pheromone binding proteins PBP1 and PBP2 are expressed in adjacent cells. Taken together, these results suggest that larvae have the same molecular repertoire as adult males use for pheromone detection.

Monika Zielonka, Priscilla Gehrke, Elisa Badeke (Schuh), Silke Sachse, Heinz Breer and Jürgen Krieger

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Built on an idea conceived by all authors.

Experimental design: M. Zielonka, E. Schuh (10 %), S. Sachse, J. Krieger

Performance and analysis of single sensillum recordings: M. Zielonka, E. Schuh (25 %)

Performance and analysis of immunohistochemistry: M. Zielonka, P. Gehrke

Wrote the manuscript: M. Zielonka, E. Schuh (10 %), S. Sachse, H. Breer, J. Krieger

Chapter 2

The sense of smell in female silkmoths: Physiological characterization of trichoid sensilla and odor-guided behavior

In this chapter I examined the sensitivity and the receptive range of OSNs of two types of trichoid sensilla (long-and medium-sized) of female *B. mori* using SSR. OSNs of both sensillum types are broadly tuned and respond mainly to host plant volatiles and acids. Since trichoid sensilla of female moths might detect both pheromone and plant volatiles, I included the mating status in the characterization. After mating, the sensitivity in medium-sized trichoid sensilla (T2) to the host plant volatiles cis-jasmone, methyl salicylate and (+)-linalool was drastically enhanced. By performing Y-maze experiments, we could additionally demonstrate that females show attraction (cis-jasmone) and aversion behavior (indole, isovaleric acid) upon odor stimulation, which leads to an increase in wing fanning activity. Furthermore, oviposition behavior is influenced by (+)-linalool. The results suggest that, although considered as stationary throughout their life, a female silkmoths triggers different behaviors dependent on odor stimulation.

Elisa Schuh, Bill S. Hansson, Silke Sachse, Sonja Bisch-Knaden

in preparation

Built on an idea conceived by all authors.

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Performance and analysis of two-choice behavioral assay: E. Schuh (100 %)

Collection and analysis of volatiles: E. Schuh (95 %), S. Bisch-Knaden

Wrote the manuscript: E. Schuh (75 %), B. S. Hansson, S. Sachse, S. Bisch-Knaden

Chapter 3

A challenge for a male noctuid moth? Discerning the female sex pheromone against the background of plant volatiles

I investigated the behavioral consequence of pheromone-plant interaction in male *Heliothis virescens*. Using odor collection techniques coupled with gas chromatography mass spectrometry and the wind tunnel assay I show in this chapter that pheromone-plant interaction occurs at the behavioral level. However, plant volatiles reduce pheromone attraction in male *H. virescens* only when adding an unnaturally high concentrated, synthetic plant compound to the female-specific pheromone. Otherwise, when presenting a natural plant odor bouquet the male is very well able to perform the same odor-guided behavior towards the pheromone source compared to the pheromone stimulation alone.

Elisa Badeke (Schuh), Alexander Haverkamp, Bill S. Hansson and Silke Sachse

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Collection and analysis of volatiles: E. Schuh (100 %)

Wrote the manuscript: E. Schuh (75 %), A. Haverkamp, B. S. Hansson, S. Sachse

Chapter 4

Electrical synapses mediate synergism between pheromone and food odors in Drosophila melanogaster

In this study we found that a food odor, namely vinegar, increases the pheromone response (cVA) in virgin female *D. melanogaster* at the level of the projection neurons (PNs) within the antennal lobe (AL), but not at the level of the OSNs. As a behaviorally consequence, vinegar enhances the receptivity in females during courtship. Moreover, in this study we demonstrate that the mechanism involved is mediated by electrical synapses between excitatory local interneurons (eLNs) and PNs.

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*These authors contributed equally to the work.

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Performance and analysis of imaging experiments: S. Das, F. Trona

Performance and analysis of photoactivation experiments: S. Das

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CHAPTER 1

Larval sensilla of the moth Heliothis virescens respond to sex pheromone components

Monika Zielonka*, Priscilla Gehrke*, Elisa Badeke (Schuh), Silke Sachse, Heinz Breer* and Jürgen Krieger

Caterpillar of *Heliothis virescens*. Photo: E. Schuh

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Larval sensilla of the moth *Heliothis virescens* respond to sex pheromone components

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Abstract

Female-released sex pheromones orchestrate the mating behaviour of moths. Recent studies have shown that sex pheromones not only attract adult males but also caterpillars. Single sensillum recordings revealed that larval antennal sensilla of the moth *Heliothis virescens* respond to specific sex pheromone components. In search for the molecular basis of pheromone detection in larvae, we found that olfactory sensilla on the larval antennae are equipped with the same molecular elements that mediate sex pheromone detection in adult male moths, including the *Heliothis virescens* receptors 6 (HR6) and HR13, as well as sensory neurone membrane protein 1 (SNMP1). Thirty-eight olfactory sensory neurones were identified in three large sensilla basiconica; six of these are considered as candidate pheromone responsive cells based on the expression of SNMP1. The pheromone receptor HR6 was found to be expressed in two cells and the receptor HR13 in three cells. These putative pheromone responsive neurones were accompanied by cells expressing pheromone-binding protein 1 (PBP1) and PBP2. The results indicate that the responsiveness of larval sensilla to female-emitted sex pheromones is based on the same molecular machinery as in the antennae of adult males.

Keywords: olfaction, moth, pheromone detection, larvae.

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Introduction

In insects, pheromones trigger or inhibit various behaviours, such as courtship, mating and aggregation (Hansson & Stensmyr, 2011; Depetris-Chauvin *et al.*, 2015; Yew & Chung, 2015). Lepidopteran species, most notably nocturnal moths, are prominent for the use of species-specific sex pheromones that are released from females to attract adult males (Schneider, 1992; Zhang *et al.*, 2015b). The antennae of adult male moths are highly specialized for sensitive detection of pheromone components in several respects. First, they carry thousands of long olfactory hairs (sensilla trichodea) that house olfactory sensory neurones (OSNs), which respond specifically to pheromones (Almaas & Mustaparta, 1991; Baker *et al.*, 2004) and express specific pheromone receptors (Sakurai *et al.*, 2004; Gohl & Krieger, 2006). Typically, OSNs – including pheromone-responsive neurones – additionally express the odorant receptor coreceptor (Orco), that is highly conserved conserved amongst different insect species (Krieger *et al.*, 2003; Larsson *et al.*, 2004). Orco is thought to form heteromers with the ligand-binding odorant receptor (OR) and functions as a cation channel that opens upon OR activation (Neuhaus *et al.*, 2005; Sato *et al.*, 2008; Wicher *et al.*, 2008). Second, the pheromone-responsive neurones express sensory neurone membrane protein 1 (SNMP1), which is considered to function as a co-receptor for pheromone receptors (Rogers *et al.*, 1997; Benton *et al.*, 2007; Vogt *et al.*, 2009) and to enhance the sensitivity of pheromone detection systems (Jin *et al.*, 2008; Li *et al.*, 2014; Pregitzer *et al.*, 2014). Third, the sensillum lymph contains pheromone-binding proteins (PBPs), which are secreted by supporting cells and supposedly mediate the transfer of pheromone molecules to the receptors (Vogt, 2003; Leal, 2013).

By definition, sex pheromones are chemical compounds that serve sexual interaction between adult male and female individuals of the same species and thus are important parameters for reproductive behaviour. In recent studies on *Spodoptera littoralis, S. exigua and Plutella xylostella*, it was unexpectedly found that the larvae (regardless of sex and stage) also respond to the main sex pheromone component and are attracted to food containing this species-specific chemical signal (Poivet *et al.*, 2012; Jin *et al.*, 2015; Zhu *et al.*, 2016). However, it is unclear how these compounds are received by the larvae, ie whether the larvae employ the same, highly specific molecular elements as found in OSNs of the male antenna, especially the elements that determine pheromone specificity, most notably the receptors for pheromone components and the putative co-receptor SNMP1. These molecular elements, which are essential to pheromone reception, have been thoroughly studied for the moth species *Heliothis virescens* (Krieger *et al.*, 2004; Gohl & Krieger, 2006; Grosse-Wilde *et al.*, 2007; Forstner *et al.*, 2008; Wang *et al.*, 2011; Pregitzer *et al.*, 2014). In adult males of *H. virescens*, about 80% of the 12 000 olfactory sensilla contain one OSN responding to the major pheromone component Z11-hexadecenal (Z11-16:Ald) (Almaas & Mustaparta, 1991; Baker *et al.*, 2004; Hillier & Vickers, 2007) and expressing the pheromone receptor, HR13 (Gohl & Krieger, 2006). About 7–15% of the sensilla contain one OSN that respond to the principal minor component Z9-tetradecenal (Z9-14:Ald) and express the pheromone receptor HR6 (Wang *et al.*, 2011). All OSNs expressing HR6 or HR13 also express the SNMP1 protein (Krieger *et al.*, 2002; Forstner *et al.*, 2008). These neurones are associated with cells that express the pheromone-binding proteins PBP1 and PBP2 (Krieger *et al.*, 2004, 2009). An interplay of these molecular elements is believed to determine the specific and sensitive reception of female-released sex pheromone components. To explore the molecular basis for the responsiveness of caterpillars to sex pheromone components, we assessed the larval antenna of *H. virescens* for molecular elements that are involved in pheromone detection by adult males, namely pheromone receptors, the membrane protein SNMP1 and pheromone-binding proteins.

Results and discussion

Morphology of the larval antennae of H. virescens

In the larvae of holometabolic moths, caterpillars, the morphology of the antenna is quite different from that of adults. The antenna of lepidopteran larvae consists of only three small segments (Fig. 1A), where the middle segment S2 and the most apical segment S3 carry a total of nine hair-like sensilla of different morphologies. Besides one sensillum styloconicum, two long sensilla chaetica, three small sensilla basiconica and three large sensilla basiconica (B1–B3) are found (Fig. 1A, B). Amongst the different sensilla types only the large sensilla basiconica are morphologically and functionally classified as olfactory hairs, whereas the other types are thought to serve gustatory or mechanosensory functions (Schoonhoven, 1987; Laue, 2000; Vogt *et al.*, 2002; Poivet *et al.*, 2012).

Response of larval antennae to pheromones

In order to assess whether sensory neurones in the larval antennae of *H. virescens* may respond to the major and minor sex pheromone components, we performed single sensillum recordings. All recordings were obtained from the B2 sensillum and the global firing activity was determined in response to Z9-14:Ald, Z11-16:Ald and the solvent (hexane). Upon application of the

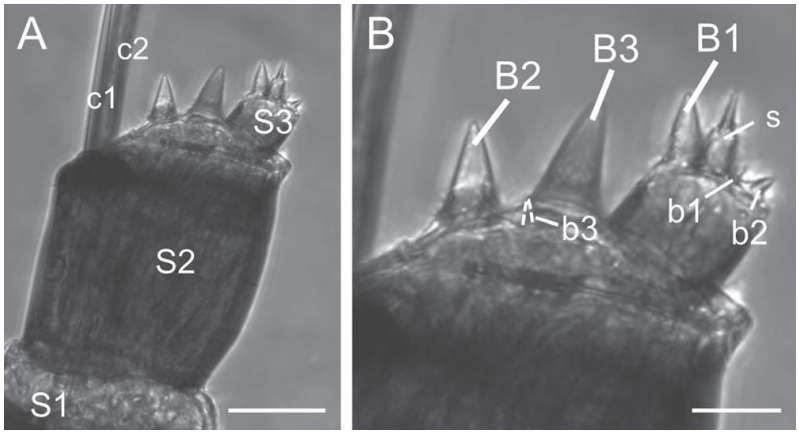

Figure 1. Organization of the larval antenna. Outer morphology of the antenna of a *Heliothis virescens* fifth-instar larva. (A) The antenna consists of three segments (S1–S3), with different sensilla types carried on S2 and S3. The two sensilla chaetica are indicated (c1, c2). (B) Higher magnification showing the upper part of S2 and S3; s, sensillum styloconicum; b1, b2, b3, small sensilla basiconica; B1, B2, B3, large sensilla basiconica (olfactory sensilla types). The B1 sensillum is located on S3 whereas the B2 and B3 sensilla are located on S2. Scale bars: A = 50 μm, B = 20 μm.

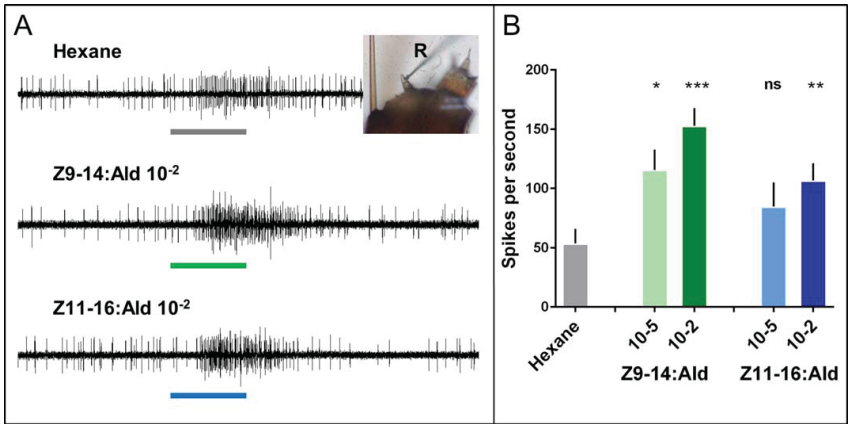

Figure 2. Single sensillum recordings from the large sensillum basiconicum B2 of the larval antenna. (A) Representative traces of single sensillum recordings showing the responses of housed sensory neurones to stimulation with the principal minor (Z9-14:Ald) and major (Z11-16:Ald) components of the female sex pheromone, as well as to the solvent (hexane). Grey, green and blue bars indicate the stimulus application (500 ms). The inset image represents a larval antenna with the recording electrode (R) inserted into the B2 sensillum. (B) Electrophysiological responses of B2 sensilla to different dilutions of the minor (green) and the major (blue) sex pheromone components. The bars represent the mean (+ SEM) global firing rate of olfactory sensory neurones (N = 9). Significant differences compared with hexane are indicated by asterisks (paired *t*-test: * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; ns, not significant).

solvent, a slight increase in the firing activity (57.7 ± 11.5 spikes/s) was observed (Fig. 2A). Application of a 10^{-5} dilution of Z9-14:Ald elicited significantly higher responses (115.5 ± 16.1 spikes/s). The responses were dose-dependent: application of higher doses (10^{-2} dilution) of the pheromone component led to a much higher response (152.8 ± 14.1 spikes/s). For the pheromone component, Z11-16:Ald, a moderate responsive activity (84.5 ± 19.2 spikes/s) was obtained at a 10^{-5} dilution, but a significant increase in the firing activity (106.5 ± 14.0 spikes/s) was measured at relatively high concentration (10^{-2} dilution) (Fig. 2B). These results indicate that sensory neurones housed in B2 sensilla are capable of responding to Z9-14:Ald and Z11-16:Ald and suggest that caterpillars of *H. virescens* can detect female-emitted sex pheromone components.

Expression of molecular elements involved in adult pheromone sensing

Following the rationale that the responsiveness of the larval antennae to pheromone components may be based on similar mechanisms as in adult males, we examined whether the pheromone receptors HR13 (major component) and HR6 (principal minor component), as well as SNMP1 and the relevant pheromone-binding proteins are expressed in larvae. Reverse transcriptase PCR (RT-PCR) experiments were performed with cDNA prepared from heads of first-instar larvae (mixed sexes) and from heads of male and female fifth-instar larvae (sexes analysed separately). Bands of the expected size were obtained for HR6, HR13 and for PBP1, PBP2 and SNMP1 in both larval stages; there was no obvious difference in band intensities between the sexes (Fig. 3). To determine whether the expression may be confined to the antennae, the larval antennae were dissected from numerous fifth-instar caterpillars and cDNA was prepared from pooled antennae. Analysis of the antennal cDNA resulted in stronger bands than with cDNA from whole heads. Experiments with primers for other members of the moth pheromone receptor family (Grosse-Wilde *et al.*, 2007), specifically HR14 and HR16, did not result in any visible bands. The integrity of the cDNA templates was confirmed with primers for the ubiquitously expressed ribosomal L31 gene (*RL31*), which resulted in intense bands for all cDNA preparations. Together, the results of the RT-PCR experiments demonstrate pronounced expression of the receptors for the major (HR13) and minor (HR6) pheromone components as well as for PBP1, PBP2 and SNMP1 in the antennae of *H. virescens* caterpillars. These findings indicate that in *H. virescens* the antennae of larvae and the antennae of adult males are equipped with the same molecular elements, which operate in pheromone detection. Moreover, the data support and extend observations made for the moth species *Spodoptera littoralis* (Poivet *et al.*, 2012) and *Bombyx mori* (He *et al.*, 2010), indicating that PBPs are expressed in larval and adult antennae. In addition, a very recent transcriptome analysis of *Manduca sexta* has provided some evidence for the expression of a candidate pheromone receptor in both the larval and the adult stages (Koenig *et al.*, 2015).

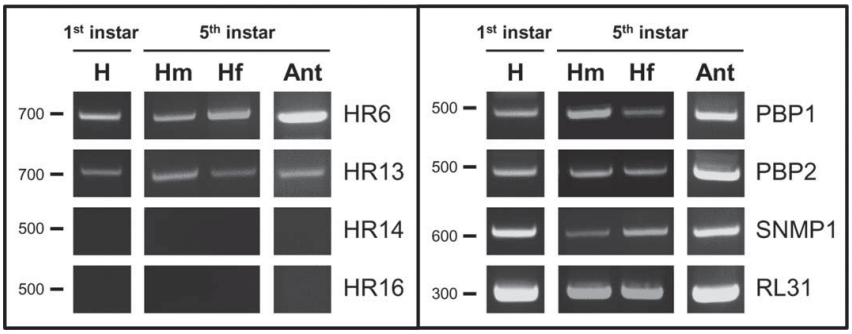

Figure 3. Expression of pheromone receptor types, pheromone-binding proteins (PBPs) and sensory neurone membrane protein 1 (SNMP1) in the larval antenna. Reverse transcriptase-PCRs were performed with cDNA from *Heliothis virescens* larvae derived either from first-instar heads (H), from fifth-instar heads of males (Hm) and females (Hf) or from pooled antennae (Ant) of fifth instars. Primer pairs were specific to the receptor for the main (HR13) and the minor (HR6) pheromone components, the pheromone receptor types HR14 and HR16, as well as for the PBPs (PBP1 and PBP2) and SNMP1. Primers for *RL31* were used as a control for the integrity of the cDNA. The positions of marker bands (in bp) are indicated on the left of the images.

Number and arrangement of olfactory sensory neurones

OSNs are characterized by the expression of the obligatory olfactory co-receptor Orco, which is required to form functional odorant receptors as well as pheromone receptors (Larsson *et al*., 2004; Ha & Smith, 2009). In order to evaluate the number and spatial organization of OSNs within the larval antenna, we performed whole mount fluorescence *in situ* hybridization (WM-FISH) experiments using a probe for Orco. Exploring the WM-FISH-treated antennae by confocal microscopy revealed a total number of 38 labelled OSNs per antenna (Fig. 4A–C, H). The Orco-positive cells were arranged in several clusters of two or three cells (Fig. 4D–G). Interestingly, in addition to the labelled somata, the dendrites of Orco-positive OSNs were also labelled. Bundles of dendrites were visible (Fig. 4A–C) that originated from cell clusters (Fig. 4D–G); however, it was not possible to optically resolve the dendrites of individual OSNs. Nevertheless, the results allowed an assignment of cell units consisting of two or three OSNs to the B1, B2 and B3 sensilla. Moreover, it was possible to determine the total number of cells within each large sensillum basiconicum (Fig. 4H). In agreement with large sensilla basiconica representing the only olfactory sensilla type on the larval antenna of moths (Laue, 2000), the projection of Orco-positive cells was restricted to this sensillum type. We found that the B1 sensillum comprised a total of eight neurones arranged in two units with three OSNs and one unit with two OSNs. The B2 sensillum housed 11 neurones grouped together in three units with three OSNs and one unit with two OSNs. The B3 sensillum contained 19 neurones organized in five units with three OSNs and two units with two OSNs. Strikingly, this pattern of spatial organization in the large sensilla basiconica of *H. virescens* (fifth instar) is in complete agreement with the results described for the larval antennae of *B. mori* (first–third instar) and *Helicoverpa assulta* (second–third instar); demonstrating that the 38 OSNs are arranged in three, four and seven olfactory units within the B1 (3/3/2 OSNs), B2 (3/3/3/2 OSNs) and B3 (3/3/3/3/3/2/2 OSNs) sensilla, respectively (Laue, 2000). Together, the data indicate a conserved number and spatial arrangement of antennal OSNs during the larval stages of different moth species. Moreover, they support the concept that the large sensilla basiconica are 'compound sensilla', ie composed of several olfactory sensillum units (clustered OSNs and supporting cells) housed in a single common cuticular hair (Laue, 2000). A similar complex organization has also been reported for larvae of Diptera and Coleoptera and is considered to be an adaptation to rapid moulting cycles (Behan & Ryan, 1978; Nicastro *et al*., 1998; Laue, 2000).

Number and topographical organization of SNMP1-expressing cells in the larval antennae

Although the functional role of SNMP1 in pheromone sensing is still under debate (Jin *et al*., 2008; Vogt *et al*., 2009; Li *et al*., 2014), the expression of SNMP1 seems to be an indication for pheromone-responsive neurones in adult male antennae (Benton *et al*., 2007; Forstner *et al*., 2008). Based on this notion, we assessed whether larval antennae contain OSNs that fulfil this criterion. The analysis of larval antennae by WM-FISH with a specific SNMP1-probe led to the labelling of several cells (Fig. 5). We found one SNMP1-labelled cell body located in segment 2 under the third segment carrying the B1 sensillum (Fig. 5A), two labelled somata at the side of the B2 sensillum (Fig. 5B) and three SNMP1-positive cells at the side of the B3 sensillum (Fig. 5C). To verify that these WM-FISH-positive cells in fact express the SNMP1 protein and project into

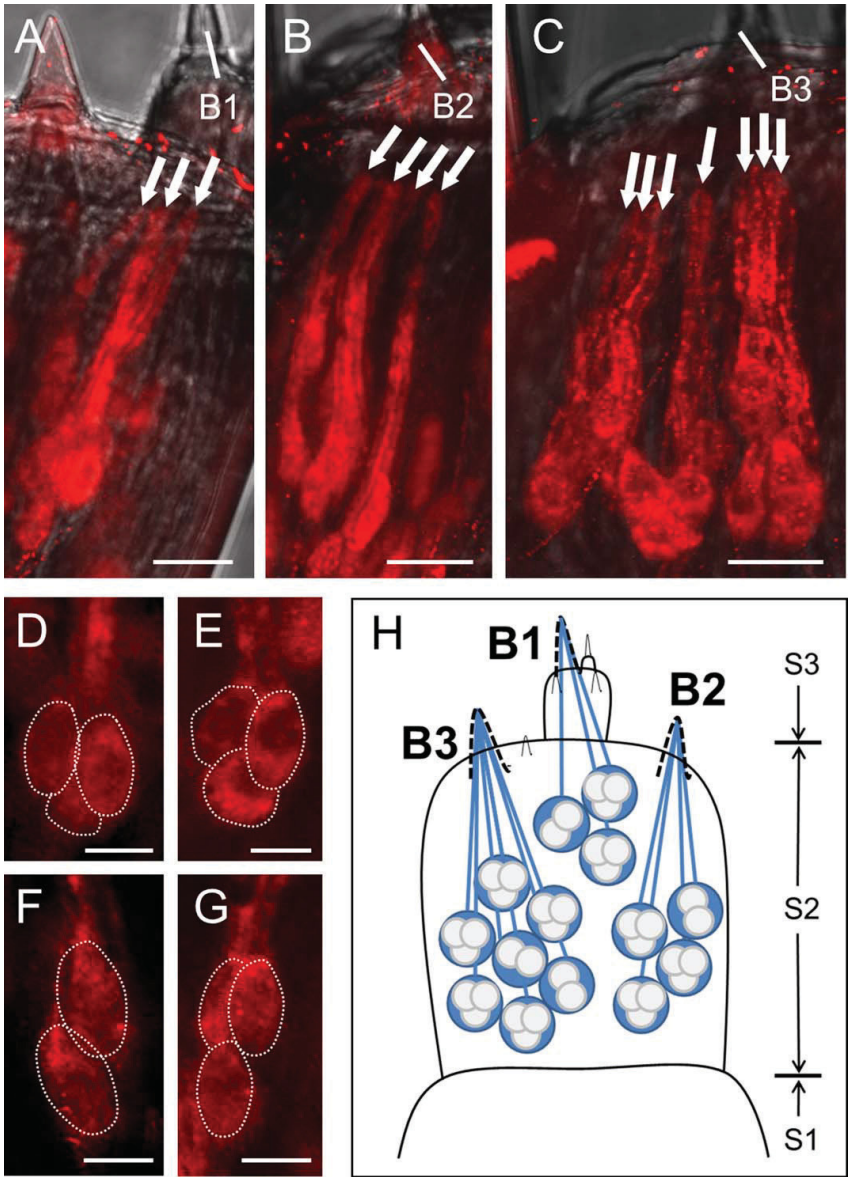

Figure 4. Arrangement of olfactory sensory neurones (OSNs) in the larval antenna. Whole mount fluorescence *in situ* hybridization with larval antennae of *Heliothis virescens* (fifth instar) and a probe for the odorant receptor coreceptor (Orco). (A–C) Orco-positive cells in the large sensilla basiconica B1 (A), B2 (B) and B3 (C), respectively. Arrows indicate dendritic bundles originating from clustered OSNs. Images represent projections of confocal image stacks from different optical layers of the larval antenna focussing on the respective sensillum. For clear data presentation images that were taken from different animals are shown. (D–G) Examples of OSN units formed by a cluster of two or three cells. (H) Schematic drawing showing the arrangement of OSNs in the antenna. OSNs cluster in units of two to three cells, with three OSN units projecting their dendrites into B1, four units housed in B2 and seven units housed in B3. S, segment. Scale bars: A–C = 20 μm, D–G = 10 μm.

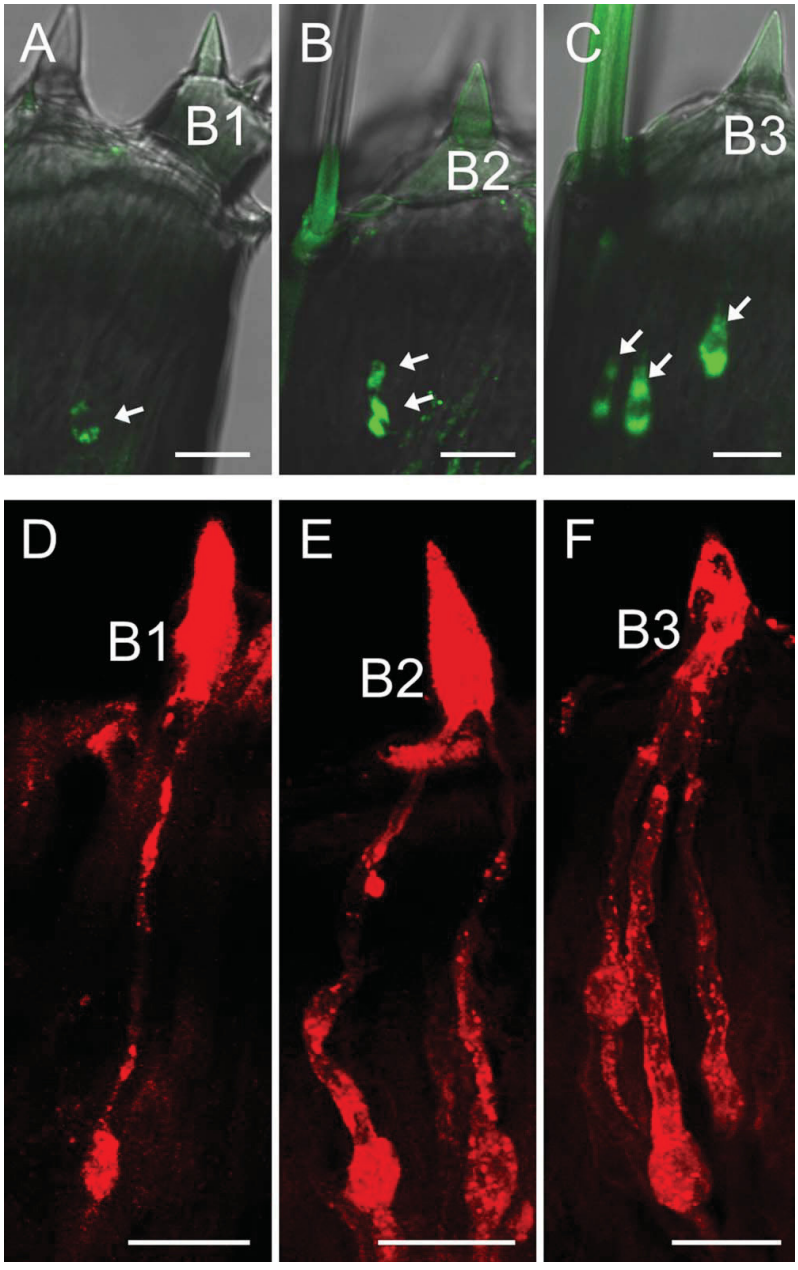

Figure 5. Topography of sensory neurone membrane protein 1 (SNMP1)-expressing cells in the larval antenna. (A–C) Whole mount fluorescence *in situ* hybridization with a larval antenna and a biotin-labelled antisense RNA probe for SNMP1; positive cells were visualized by green fluorescence. The fluorescence channel has been overlaid with the transmitted light channel. (A) The cell body of a single SNMP1-positive cell located in antennal segment 2 (S2) below the third segment carrying the large sensillum basiconicum B1. (B) Two SNMP1-labelled somata in S2 on the side of the B2 sensillum. (C) Three SNMP1-positive cells in S2 on the side of the B3 sensillum. (D–F) Immunolocalization of the SNMP1 protein. Whole mount fluorescence immunohistochemistry with larval antennae using an antibody specific for SNMP1. Immunoreactivity was visualized by an Alexa568 secondary antibody. One (D), two (E) and three (F) neurones that express SNMP1 project their dendrites into the B1, B2 and B3 sensilla, respectively. Images represent projections of selected planes from confocal image stacks of the larval antenna displaying the area around the sensillum indicated. (D), (E) and (F) show representative pictures obtained from different antennae. Scale bars = 20 μm.

large sensilla basiconica we used an SNMP1 antiserum (Rogers *et al.*, 1997) that specifically reacts with SNMP1 of *H. virescens* (Pregitzer *et al.*, 2014). In whole mount fluorescence immunohistochemistry (WM-FIHC) studies, cell bodies were strongly labelled; in addition, the dendrites of six neurones were visible. One, two and three SNMP1-positive cells projected into the B1, B2 and B3 sensillum, respectively (Fig. 5D–F), thus confirming and extending

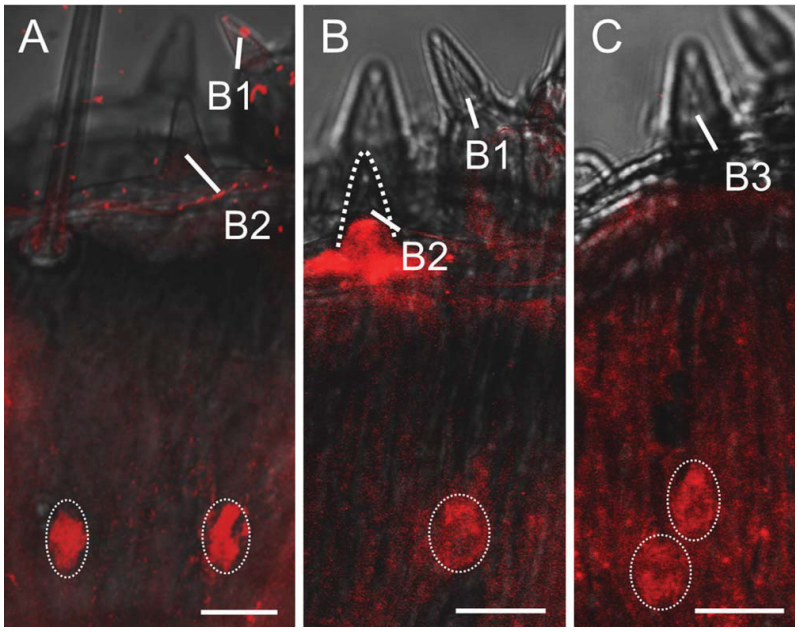

Figure 6. Localization of cells expressing pheromone receptors in the larval antenna. Whole mount fluorescence *in situ* hybridization with larval antennae using digoxigenin-labelled antisense RNA probes for HR6 (A) and HR13 (B and C). (A) The cell bodies of two HR6-expressing cells located in the S2 segment of the antenna on the sides of the large sensilla basiconica B1 and B2. (B) HR13 expression in one cell located in S2 on the sides of the B1 and B2 sensilla of the antenna. (C) Two HR13-expressing cells in S2 on the side of the B3 sensillum. Images represent projections of selected planes from confocal image stacks. Images in (B) and (C) represent two different antennae. Scale bars = 20 μm.

the results of the WM-FISH analyses (Fig. 5A–C). We found that SNMP1 expression was restricted to one to three OSNs of a single olfactory hair. This was further verified by double WM-FISH experiments with Orco- and SNMP1 probes, visualizing Orco and SMNP1-positive cells accompanied by cells that were positive only for Orco (Fig. S1). Hence, within an individual sensillum OSNs expressing SNMP1 are co-localized with OSNs that do not express this protein. Such an arrangement of SNMP1-positive cells in larval sensilla is reminiscent of the sensilla on the antenna of adult male moths (Forstner *et al.*, 2008; Gu *et al.*, 2013; Zhang *et al.*, 2015a). Taken together, in the larval antenna six out of 38 OSNs express the SNMP1-protein. Assuming that the expression of SNMP1 is indicative of pheromone responsiveness, this result would mean that six OSNs in the larval antenna are dedicated to pheromone reception. Regarding this assumption, we next investigated the expression of the receptors HR13 and HR6, the receptors for the main and the minor component of the *H. virescens* sex pheromone, respectively.

Visualization of cells expressing pheromone receptors

Performing WM-FISH experiments with a probe for the receptor HR6 we found that two cell somata were labelled; these were located within the S2 segment on the side of the B1 and B2 sensilla (Fig. 6A). Based on its position, one of the HR6-positive cells was assigned to the B2 sensillum, whereas for the second HR6-positive cell, a clear assignment to either the B1 or the B2 sensillum was not possible. Corresponding experiments with a HR13 probe led to weak, but unambiguous, labelling of one HR13-positive cell on the side of B1/B2 (Fig. 6B) and two HR13-positive cells on the side of B3 (Fig. 6C). Based on the topographical localization of the HR6- and HR13-positive cells, the two receptor types are most likely expressed in different OSNs. Thus, in each larval antenna five out of 38 antennal OSNs seem to be tuned to the detection of the female sex pheromone components. The number of five receptors expressing OSNs correlates quite well with the number of six SNMP1 expressing OSNs and suggests a co-expression of receptors and SNMP1.

Co-expression of pheromone receptors and SNMP1

Our approach to exploring possible co-expression of receptors and SNMP1 in the larval antennae using a whole mount experiment gave no clear results; therefore, tissue sections through the antennae were analysed by double FISH experiments. This procedure had previously allowed the demonstration of co-expression of receptors and SNMP1 in the antenna of adult males of *H. virescens* (Forstner *et al.*, 2008). An assessment of the tissue sections allowed visualization of HR6- and SNMP1-

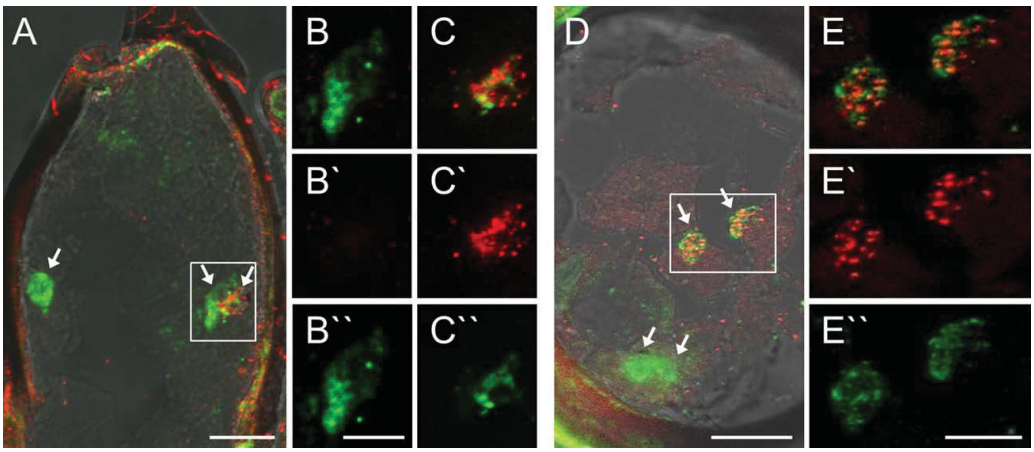

Figure 7. Co-expression of HR6 and sensory neurone membrane protein 1 (SNMP1) in the larval antenna. Double fluorescence *in situ* hybridization on a longitudinal (A) and a horizontal (D) cryosection through segment 2 of a larval antenna using differentially labelled riboprobes for HR6 (digoxigenin) and SNMP1 (biotin). (A) Projection of confocal images (spanning 10 μm) indicating three SNMP1-positive cells (green, arrows). One of these cells is also positive for HR6 (red); the red and green fluorescence channels have been overlaid with the transmitted-light channel. (B, B` and B``) Higher magnification of the area boxed in (A) showing the overlaid red and green (B), only the red (B`) and only the green (B``) fluorescence channels for the first 4 μm of the projection. (C, C` and C``) Corresponding pictures for the following 6 μm of the projection. (D) Co-expression of HR6 (red) in two of four SNMP1-positive cells (green, arrows); projection of confocal images, with the red and green fluorescence channels overlaid with the transmitted-light channel. (E, E`, E``) Higher magnification of the area boxed in (D) displaying the overlaid red and green, only the red or only the green fluorescence channels, respectively. Scale bars: A and D = 20 μm; B, C and E = 10 μm.

expressing cells on the same section (Fig. 7). Analyses of longitudinal (Fig. 7A–C) as well as horizontal sections (Fig. 7D, E) by confocal microscopy revealed that the HR6-positive cells were strictly co-labelled by the SNMP1-probe, indicating expression in the same cells. In addition, on the same section several SNMP1-positive cells were visible that did not express HR6 (Fig. 7A, D). These cells could possibly express the HR13 receptor. The data indicate co-expression of pheromone receptors and SNMP1 in OSNs of the larval antenna; a further feature that is reminiscent of the male antennae of adult *H. virescens* (Krieger *et al.*, 2002; Forstner *et al.*, 2008) and indicates that OSNs in larvae and in adults are equipped with the same molecular elements for sex pheromone detection.

PBP1- and PBP2-expressing cells in the larval antenna

In the antennae of adult male moths, OSNs that express SNMP1 and pheromone receptors are accompanied by supporting cells that co-express the two binding proteins PBP1 and PBP2 (Grosse-Wilde *et al.*, 2007; Forstner *et al.*, 2008). In both the antennae of adults and larvae, the morphology of the OSNs and the supporting cells is quite different; in larvae the supporting cells are larger and have a more flat and extended shape (Gnatzy *et al.*, 1984; Laue, 2000). In order to determine the cellular expression of PBP1 and PBP2 in the larval antennae of *H. virescens*, double WM-FISH experiments were performed. Experiments with the digoxigenin (DIG)-labelled PBP1 probe led to weak but reliable labelling of large, flat and extended cells (Fig. 8A, red); similar results were obtained with the biotin-labelled PBP2 probe (Fig. 8B, green). In the overlay of the WM-FISH signals (Fig. 8C) it was apparent that both PBP probes labelled the same cells, indicating co-expression of the two PBP types. In order to improve the visualization of PBP-expressing cells double WM-FISH experiments were performed with DIG-labelled probes for both PBP1 and PBP2. This approach resulted in much stronger labelling of PBP-expressing cells; also large unstained areas became visible, which appear to represent unstained cell nuclei (Fig. 8D). Nucleus staining with propidium iodide confirmed this assumption (Fig. 8E). In addition to the elongated nuclei of the PBP-positive cells (∼ 20 μm long; Fig. 8E, F), also small and round nuclei (diameter about 5 μm) were visible, which probably represented nuclei of sensory neurones (Fig. 8G). By confocal scanning through the whole antenna, we localized several PBP-positive cells at the periphery of the antennal cylinder (Fig. 8A–D) and lower numbers in the middle part of the antenna (Fig. 8E–F). Based on the localization of the nuclei it appeared that large nuclei surround clusters of smaller nuclei (Fig. 8G). This arrangement of OSNs and PBP-expressing supporting cells is

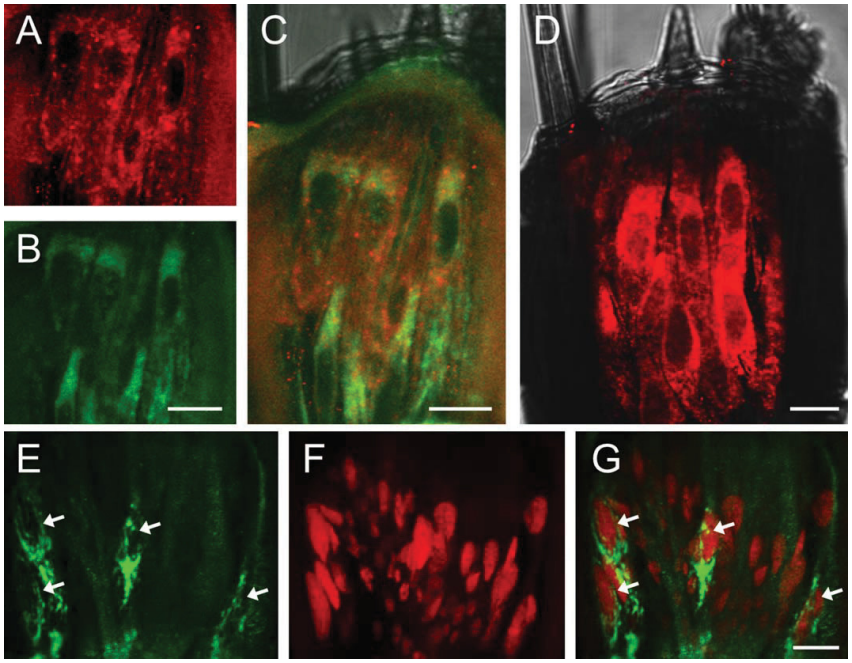

Figure 8. Visualization of pheromone-binding protein 1 (PBP1)- and PBP2-expressing cells in the larval antenna. (A–C) Differentially labelled antisense RNA probes for PBP1 (digoxigenin, DIG) and PBP2 (biotin) were used in double whole mount fluorescence *in situ* hybridization (WM-FISH) with larval antennae. PBP1- and PBP2-positive cells were visualized by detection systems leading to red and green fluorescence, respectively. (A) Red fluorescence channel showing PBP1 expression. (B) Green fluorescence channel showing PBP2 expression. (C) Overlay of the red, green and transmitted light channels. PBP1 and PBP2 label the same cells in antennal segment two indicating co-expression of the PBPs. (D) Double WM-FISH using DIG-labelled probes for PBP1 and PBP2 leading to stronger red fluorescence and clearer labelling of PBP-expressing cells. The image represents a projection of optical planes ranging from about 15–20 μm below the antennal surface. The red fluorescence channel is overlaid with the transmitted light channel. (E, F) WM-FISH with a biotin-labelled probe for PBP1 (green) and nucleus staining with propidium iodide (red). A plane lying more to the centre of the antenna is shown. (E) Green fluorescence channel visualizing PBP1-expressing cells. (F) Red fluorescence channel showing large and small nuclei. (G) Overlaid red and green fluorescence channels. Arrows mark cells with very large nuclei (red) expressing PBP1 (green). Scale bars = 20 μm.

reminiscent of sensilla on the adult male antenna (Grosse-Wilde *et al.*, 2007; Forstner *et al.*, 2009) and is in line with scanning electron microscope analysis of the larval antenna from *B. mori*, demonstrating a close association of OSNs and supporting cells in the large sensilla basiconica (Laue, 2000).

Conclusion

In the larval antennae of *H. virescens* a small subset of OSNs housed in the large sensilla basiconica express receptors tuned to sex pheromones as well as the SNMP1-protein. These OSNs are adjacent to cells that express pheromone-binding proteins that are believed to transfer pheromones towards the dendrites of the neurones. Together with the finding that sex pheromone components activate cells in the large sensilla basiconica, our data suggest that larvae and adult males use the same molecular elements and mechanisms to respond to components of the female-released sex pheromone. These findings confirm and extend the results of previous behavioural studies demonstrating that female sex pheromones are not only perceived by adult male moths but also by larval stages (eg Poivet *et al.*, 2012).

Experimental procedures

Animals and tissue collection

Heliothis virescens larvae were kindly provided by Bayer CropScience, Frankfurt, Germany. For total RNA isolation, antennae or complete heads of first- or fifth-instar larvae were dissected from cold anaesthetized larvae and collected in 1.5-ml reaction tubes cooled with liquid nitrogen. For sex determination of larvae we examined their gonads by dissecting the abdomen. For whole mount *in situ* hybridization experiments antennae were dissected and transferred directly to 0.2-ml reaction tubes filled with fixation solution (see below).

Single sensillum recordings

For electrophysiological recordings a larva was mounted in half a 1 ml pipette tip with the head on the narrow end. To prevent any movement the larva was fixed with Parafilm (Pecheney Plastics Packaging, Chicago. IL) and covered in such a way that only one antenna was protruding out of the Parafilm. The pipette tip was fixed with wax on an object slide. A silver electrode (reference electrode) was inserted into the abdomen of the larva. A tungsten electrode (recording electrode) was electrolytically sharpened by immersing the tip into a 10% KNO_2 solution and was positioned into the B2 sensillum of the larval antenna using a micromanipulator (Luigs and Neumann, Ratingen, Germany), obtaining a stable electrical contact with a high signal-to-noise ratio. Extracellular signals from ORNs were amplified 10× and digitally converted via a USB-IDAC (Syntech, Kirchzarten, Germany) connection to a computer (sampling rate 10.667 Hz). Action potentials were visualized and analysed using Syntech AutoSpike 32 software (version 3.9). Neurone activities were recorded for 30 s, starting 10 s before a stimulation of 0.5 s. Neuronal activity was analysed by determining the number of spikes for 25 ms bins over a time window of 1 s before and 1 s after stimulation. As single neurones could not be distinguished, the total response from all neurones in the sensillum was used for analysis. Responses of the sensillum were defined as the increase in the action potential frequency (spikes/s) of all corresponding neurones by calculating the difference of the highest responses within the time windows after and before stimulation. For statistical analysis the pheromone responses were compared with the responses to hexane using a paired *t*-test with GraphPad Prism 6 (GraphPad Software, Inc. La Jolla, CA).

Odour stimuli

The *H. virescens* minor sex pheromone component Z9-tetradecenal (Z9-14:Ald, CAS 53939-27-8, purity > 93%) and the major sex pheromone component Z11-hexadecenal (Z11-16:Ald, CAS 53939-28-9, purity 97–98%) were acquired commercially from Pherobank (Wijk bij Duurstede, The Netherlands, http://www.pherobank.com). Pheromone compounds were dissolved in hexane to obtain 10^{-2} and 10^{-5} dilutions. Either a pheromone dilution or hexane as the control (6 µl each) was pipetted onto a piece of filter paper placed inside a Pasteur pipette. Subsequently, the pipette tip was inserted into a delivery tube through a hole positioned at around 10 cm distance from the preparation. For odour application, a stimulus controller was used that produces a 0.5-s air-puff that passes through the stimulus pipette into a humidified continuous air stream (0.5 l/min). In order to exclude mechanical artefacts a compensatory flow was interposed when the antenna was not stimulated. The stimulus outlet was placed at a distance of 2 cm from the mounted larva.

RT-PCR

Total RNA from heads of *H. virescens* larvae (fifth instar) were isolated using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) following the recommendation of the supplier. For first-instar heads and fifth-instar antennae a mixture of male and female tissue was collected and total RNA isolated using a NucleoSpin RNA kit (Machery and Nagel, Düren, Germany) following the protocol recommended by the supplier. This protocol included a DNase treatment of the RNA preparation to eliminate possible genomic DNA contamination. Male and female tissues from fifth-instar larval heads were analysed separately. Poly $(A)^+$ RNA was isolated from total RNA with oligo $(dT)_{25}$ magnetic dynabeads (Thermo Scientific, Waltham, MA) and transcribed into cDNA as previously described (Krieger *et al.*, 2002). In RT-PCR experiments with specific primer pairs we used the following PCR conditions: 1 min 40 s at 94 °C, then 21 cycles of 94 °C for 30 s, 55 °C for 40 s and 72 °C for 2 min, with a decrease of the annealing temperature by 0.5 °C per cycle. Subsequently, 19 further cycles under the same conditions as the last cycling step were performed, followed by incubation for 7 min at 72 °C.

The primer pairs used for the specific amplifications were: Orco, 5′-CAC TGT CCT GTT CTT CGC-3′ and 5′-GCT CAG TTC CAT GAG GGG-3′; HR6, 5′-AGT AAC TTG GCC GCA GGA AA-3′ and 5′-CAT AGC CTT CAC ATG AAC CG-3′; HR13, 5′-CGG TCT ACT TAC TCG GCT TGG-3′ and 5′-CTG TGC GAC TGT CTG AGC ATC-3′; HR14, 5′-GTT CAC ACT GTA CCT CAC TGG-3′ and 5′-GAA CAA CAT TGG CCC GAA TAC-3′; HR16, 5′-CGA GAC CAA GTT CCA AAG TGG-3′ and 5′-AGG TCT TCA AAA TCG CAG CC-3′; SNMP1, 5′-CGA CGT GTT CTA CTT TAA CCC-3′ and 5′-TTG GCA AAG TCT CCG ATG TT-3′; PBP1, 5′-GGA ATT CCA TAT GTC GCA AGA TGT TAT GAA GAA CCT G-3′ and 5′-AGA CAC TCG AGT TCC TAA ACT TCG GCC AAG AC-3′; PBP2, 5′-GGA ATT CCA TAT GTC CAA AGA ACT GCT CAC AAA GAT G-3′ and 5′-AGA CAC TCG AGC ATC TAC GCG GCA GTC ATG ATC-3′. To verify the integrity of the different cDNAs the primer pair 5′-CAA CGA AGT TGT AAC TCG TG-3′ and 5′-TTC TTG GCT AGC GTC CAC AT-3′ was used for the amplification of the ubiquitously expressed *RL31* gene. PCR products were analysed by agarose gel electrophoresis.

WM-FISH

Single or double WM-FISH were performed as described previously (Qiao *et al.*, 2010; Schultze *et al.*, 2012) with a few modifications. Dissected antennae were fixed in 4% paraformaldehyde in 0.1 M $NaCO_3$, pH 9.5, 0.03% Triton X-100 (Sigma Aldrich, St. Louis, MO) for 24 h at 6 °C. Subsequently antennae were washed for 1 min in phosphate-buffered saline (PBS = 145 mM NaCl, 1.4 mM KH_2PO_4, 8 mM Na_2HPO_4, pH 7.1) containing 0.03% Triton X-100, then incubated for 10 min in 0.2 M HCl, 0.03% Triton X-100 and washed for 2 min in PBS. Following this step, antennae were incubated for 10 min in acetylation solution (0.1 M triethanolamine supplemented with acetic anhydride before use to give a final concentration of 0.25% acetic anhydride) and washed three times for 3 min each time in PBS. For the detection of PBPs and HR13 we used an alternative fixation method with ZnFA solution (0.25% $ZnCl_2$, 1% formaldehyde, 135 mM NaCl, 1.2% sucrose, 0.03% Triton X-100) for 24 h at room temperature. Afterwards antennae were washed three times for 15 min each time in HBS (HEPES-buffered saline) buffer (150 mM, NaCl, 5 mM KCl, 25 mM sucrose, 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 5 mM $CaCl_2$, 0.03% Triton X-100). For prehybridization antennae were incubated for 30 min at 55 °C in whole

mount *in situ* hybridization solution (WM-HBL; 50% formamide, 5× SSC (sodium chloride/sodium citrate), 1× Denhardt's reagent, 50 μg/ml yeast RNA, 1% Tween 20, 0.1% 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (Chaps), 5 mM ethylenediaminetetraacetic acid, pH 8.0). Antennae were then hybridized with WM-HBL containing specific DIG-labelled and/or biotin-labelled antisense RNA probes at 55 °C for 3–4 days. After washing four times for 15 min at 60 °C in 1× SSC solution with 0.03% Triton X-100, antennae were incubated in blocking solution [1% blocking reagent (Roche, Indianapolis, IN, USA) in Tris buffered saline (TBS; 100 mM Tris, pH 7.5, 150 mM NaCl), 0.03% Triton X-100] overnight at 4 °C. The antennae were then incubated with an anti-DIG alkaline phosphatase-conjugated antibody (Roche) diluted 1:500 in blocking solution for the detection of DIG-labelled RNA probes; for detection of biotin-labelled probes a streptavidin horseradish peroxidase-conjugate (1:100, tyramide signal amplification (TSA) kit, PerkinElmer, Boston, MA, USA) was used. After incubation for 3 days at 4 °C, antennae were washed five times for 10 min each time in TBST (TBS with 0.05% Tween 20) at room temperature. For visualization of DIG-labelled probes the antennae were subsequently incubated in 2-hydroxy-3-naphtoic acid-2'-phenylanilide phosphate (HNPP) solution [Roche; 1:100 in DIG alkaline phosphatase (DAP)-buffer (100 mM Tris, pH 8.0, 100 mM NaCl, 50 mM $MgCl_2$)] for at least 7 h or overnight (17 h) at 4 °C in the dark. Biotin-labelled probes were visualized using components of the TSA Fluorescein System and incubation with a 1:50 or a 1:100 dilution for 17–24 h at 4 °C in the dark. For double WM-FISH, antennae were first treated with HNPP solution and then incubated with the TSA components, with a washing step of three times for 10 min each time with TBST included between the substrate treatments. Finally, antennae were washed three times for 10 min each time in TBST. In the case of nuclei staining, antennae were incubated for 1 h in propidium iodide diluted 1:1000 in TBS and then washed three times for 5 min each time in TBST. Finally, the antennae were rinsed in PBS and mounted in mowiol solution (10% polyvinylalcohol 4-88, 20% glycerol in PBS).

WM-FIHC

WM-FIHC was performed as described previously (Schultze *et al.*, 2013) with some modifications. Antennae were dissected and fixed in ZnFA solution (0.25% $ZnCl_2$, 1% formaldehyde, 135 mM NaCl, 1.2% sucrose, 0.03% Triton X-100) for 24 h at room temperature. The antennae were then washed three times for 15 min each time in HBS buffer (150 mM, NaCl, 5 mM KCl, 25 mM sucrose, 10 mM HEPES, 5 mM $CaCl_2$, 0.03% Triton X-100) and incubated in 80% methanol/20% dimethyl sulphoxide (DMSO) for 1 h. Subsequently, antennae were washed for 5 min in 0.1 M Tris pH 7.4, 0.03% Triton X-100 and incubated in blocking solution [PBS, 5% normal goat serum (Dianova, Hamburg, Germany), 1% DMSO, 0.03% Triton X-100] for at least 3 h or overnight. The blocking solution was replaced by a primary antibody solution consisting of the anti-*Antheraea polyphemus* SNMP1 (Anti-ApolSNMP1) antiserum (Rogers *et al.*, 1997) diluted 1:100 in blocking solution. Antennae were subsequently placed in a water bath sonifier (Bransonic 1200, Branson, Danbury, CT, USA) for 30 s followed by incubation for 4 days at 4 °C. Antennae were then washed three times for 15 min each time in PBS, 1% DMSO, 0.03% Triton X-100 and after a 30-s sonification step were incubated in blocking solution containing an anti-rabbit Alexa568 coupled secondary antibody (Invitrogen, dilution 1:1000) for 3 days at 4 °C in the dark. Finally, antennae were washed three times for 15 min each time in PBS with 1% DMSO, 0.03% Triton X-100, briefly rinsed in PBS and then mounted in mowiol solution.

Fluorescence in situ *hybridization on cryosections*

Double-FISH was performed on sections using protocols described earlier (Krieger *et al.*, 2002, 2004). Cryosections of heads of fifth-instar larvae were fixed for 30 min at 4 °C using 4% paraformaldehyde in phosphate buffer and washed at room temperature for 1 min in PBS, for 10 min in 0.2 M HCl and for 2 min in PBS with 1% Triton X-100 followed by two 30-s washes in PBS. Slides were then incubated for 10 min in 50% formamide, 5× SSC. In all subsequent incubation steps slides were placed in a humid box containing filter paper soaked with 50% formamide (for hybridization) or H_2O (all other steps). Sections were incubated with a DIG-labelled HR6 antisense RNA probe and a biotin-labelled SNMP1 antisense RNA probe diluted in hybridization buffer at 55 °C overnight. Posthybridization, sections were washed twice for 30 min in 0.1× SSC at 60 °C, then treated for 30 min with blocking solution (see WM-FISH section above) and incubated for 1 h at 36 °C with an anti-DIG AP-conjugated antibody (Roche) diluted 1:500 in blocking solution for the detection of DIG-labelled RNA probes and a streptavidin horseradish peroxidase-conjugate (1:100, TSA kit, PerkinElmer) for detection of biotin-labelled probes. After washing three times for 5 min each time in TBST, the antennae were incubated in HNPP solution (Roche; 1:100 in DAP-buffer) for 50 min at room temperature for the visualization of DIG-labelled probes. This was followed by three washes for 5 min each with TBST. Biotin-labelled probes were visualized using components of the TSA Fluorescein System and incubation with a 1:100 dilution for 50 min at room temperature. After three final washes for 5 min each in TBST, sections were embedded in mowiol solution.

Analysis of antennal sections by microscopy

Antenna from WM-FISH and WM-FIHC experiments were analysed on a Zeiss LSM 510 meta laser scanning microscope (Zeiss, Oberkochen, Germany). Confocal image stacks of the red and green fluorescence channels as well as the transmitted-light channel were recorded from the antennae. Image stacks were used to generate projections of selected optical planes, with the fluorescence and transmitted light channels overlaid or shown separately.

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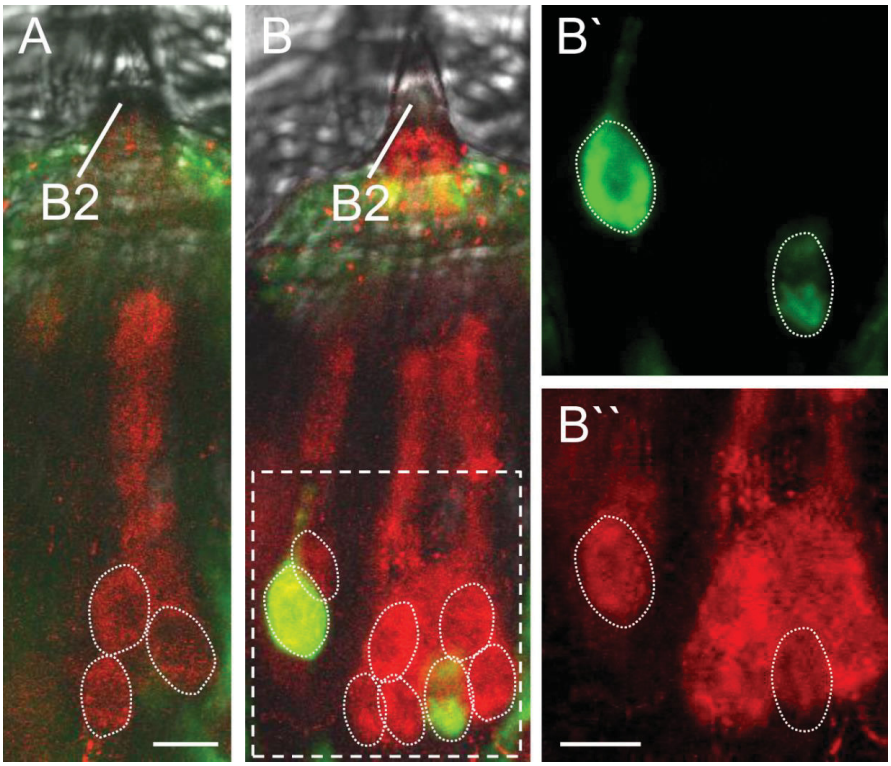
Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Figure S1. Co-expression of the odorant receptor coreceptor (Orco) and sensory neurone membrane protein 1 (SNMP1) in the larval antenna. (A, B) Differentially labelled antisense RNA probes for Orco (digoxigenin) and SNMP1 (biotin) were used in a double whole mount fluorescence *in situ* hybridization with a larval antenna to visualize Orco- and SNMP1-positive cells. Two different optical planes covering all units belonging to the large sensillum basiconicum B2 are shown. (A) One unit with three Orco-positive cells. (B) Three units (one × two cells and two × three cells) showing co-expression of SNMP1 and Orco in two cells (yellow) belonging to different units. (B′ and B″) Higher magnification of the area boxed in (B) with the green fluorescence channel (B′) showing two SNMP1-positive cells and the red channel (B″) showing Orco-positive cells; the circled areas mark SNMP1-positive cells. Scale bars = 10 μm.



Supplementary Figure S1
Co-expression of Orco and SNMP1 in the larval antenna.
A-B: Differentially labeled antisense RNA probes for Orco (DIG) and SNMP1 (biotin) were used in a double WM-FISH with a larval antenna to visualize Orco- and SNMP1-positive cells. Two different optical planes covering all units belonging to B2 sensillum are shown. **A:** One unit with 3 Orco-positive cells. **B:** Three units (1 x 2 cells and 2 x 3 cells) showing co-expression of SNMP1 and Orco in 2 cells (yellow) belonging to different units. **B`** and **B``:** Higher magnification of the area boxed in B with the green fluorescence channel **(B`)** showing 2 SNMP1-positive cells and the red channel **(B``)** showing Orco-positive cells, the circled areas mark SNMP1-positive cells. Scale bars: 10 µm.

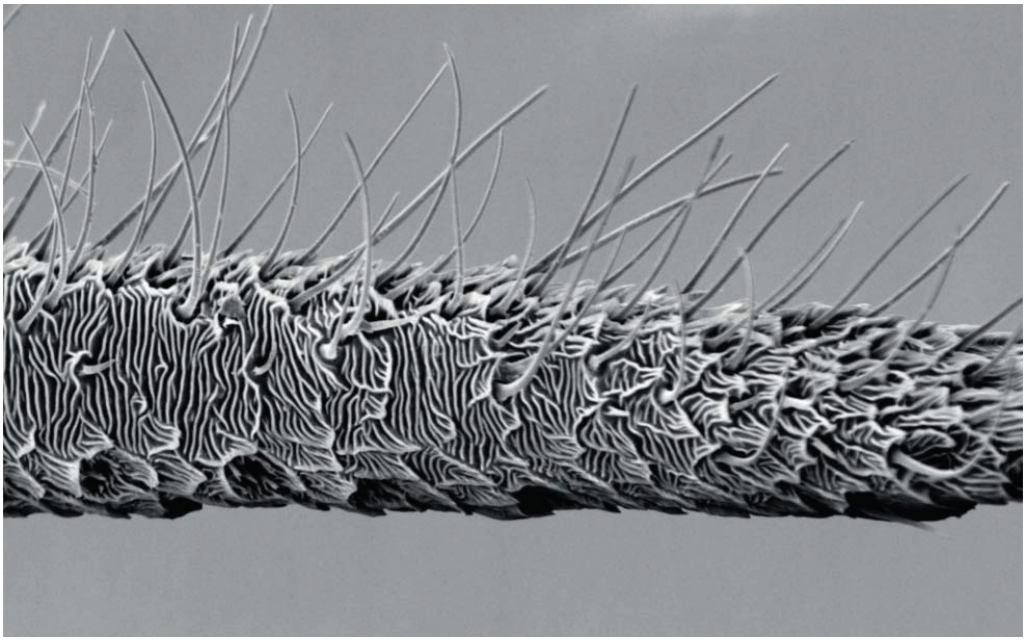
CHAPTER 2

The sense of smell in female silkmoths: Physiological characterization of trichoid sensilla and odor-guided behavior

Elisa Schuh, Bill S. Hansson, Silke Sachse, Sonja Bisch-Knaden

'No doubt, you can understand my excitement, when I saw a deflection of the electron beam of my oscilloscope, lasting for the time of the stimulus. I praised the chemists to give me such a powerful extract since I thought, naively, that the natural emanation of a fresh female gland would never suffice to elicit a visible response in my recording system.'

Dietrich Schneider, Insect pheromone research: some history and 45 years of personal recollections



Scanning electron micrograph of an antennal branch including the sensilla of a *Bombyx mori* female. Photo: S. Bisch-Knaden

The sense of smell in female silkmoths:

Physiological characterization of trichoid sensilla and odor-guided behavior

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ABSTRACT

The olfactory organs of moths are often sexually dimorphic. Male antennae possess mainly one type of sensory hairs, so-called long trichoid sensilla, housing olfactory sensory neurons tuned to the female's sex pheromone, while female antennae usually lack this sensillum type. Female silkmoths, *Bombyx mori*, however, have numerous long trichoid sensilla, described to house two neurons that detect linalool, and benzoic acid, respectively. Medium-sized trichoid sensilla represent the second most common sensillum type at the female silkmoths' antenna with a so far unknown receptive range. In our study, we aimed at characterizing both trichoid sensillum types in detail, i.e. we performed single sensillum recordings with a large set of ecological relevant and chemically diverse odorants. We found that long trichoid sensilla respond to the already known ligands, and in addition to several chemically related compounds. The most attractive host plant volatile for silkmoth larvae, cis-jasmone, especially activated medium-sized trichoid sensilla. After mating, only neurons housed in medium-sized trichoid sensilla responded considerably more sensitive to their ligands, indicating that these sensilla play a role in finding a suitable oviposition site. We furthermore established a behavioral assay, the Y-maze, in order to investigate the significance of relevant odorants. By the usage of the best ligands of both trichoid sensillum types cis-jasmone was the only odorant that attracted mated female moths, showing that cis-jasmone is a key odorant in finding host plants not only for silkmoth larvae but also for female adults. Odorants being detected by long trichoid sensilla, however, were not attractive in the Y-maze, but elicited aversion behavior. Therefore, long trichoid sensilla of female silkmoths might not be involved in host search but might be important in a different behavioral context.

Keywords: *Bombyx mori*, trichoid sensilla, GC-MS, single sensillum recording, choice-assay, linalool, cis-jasmone, acids

INTRODUCTION

Nocturnal insects like moths heavily rely on their sense of smell. Male moths use the female-produced sex pheromone to find mating partners, while female moths locate suitable egg-laying sites by plant-released volatiles. These different, sex-specific tasks are often reflected in a sexual dimorphism of the moths' antenna (reviewed in Koontz and Schneider1987 and Rospars1988): males have thicker or more complex antennae than females (Steinbrecht1970), covered mainly with a high number of a specialized type of sensory hairs, so-called long trichoid sensilla (Clyne et al.1997, Schneider1956). These sensilla house olfactory sensory neurons that are tuned to detect the female's sex pheromone (Kaissling and Priesner1970, Kaissling et al.1978). The female moths' antenna is usually structurally simpler and lacks long trichoid sensilla (Scheffler1975). However, the females also possess sensory hairs of several types that house olfactory sensory neurons detecting plant-related odors (Shields and Hildebrand2001).

B. mori is a domesticated moth bred for silk production, and has become a model organism in biology. So far, most of the research in *B. mori* is focused on males and their ability to sense and process female sex pheromones (Butenandt1959, Schneider1956) as females are considered to be anosmic for their own pheromone (Schneider1957). The sexual dimorphism in *B. mori* is not that distinct compared to e.g. *L. dispar* (Scheffler1975). The antennae of both males and females are branched (Fig. 1) and trichoid sensilla are the most abundant sensillum type on the antenna of silkmoths. They may comprise 80% of the olfactory sensilla in males, and as much as 69% in females (Steinbrecht1970). Based on their length, they are divided into a long (male: 100 µm, female: 90 µm) and two medium sized (40 - 50 µm) morphological types of trichoid sensilla (Steinbrecht1970, Steinbrecht1973). Each long trichoid sensillum in *B. mori* houses two neurons irrespective of the sex, whereas the morphological medium-sized trichoid sensillum types house either two, or up to three neurons. The two neurons, which are housed in long-sized trichoid sensilla are of different thickness, whereas neurons of medium-sized trichoid sensilla are reported to be morphologically similar in size (Steinbrecht, 1973). Steinbrecht also demonstrated that all neurons described, irrespective of the sensillum type are unbranched.

Male silkmoths have three times more long trichoid sensilla, and have only half the number of medium-sized trichoid sensilla than females (Steinbrecht1970). One of the two neurons being housed in long-sized trichoid sensilla of males is activated by the female sex pheromone bombykol, while the second one is highly sensitive to bombykal, a putative minor pheromone component (Kaissling and Priesner1970, Kaissling et al.1978). In addition, bombykal is a behavioral antagonist of bombykol for male *B. mori*, and moreover is a sex pheromone component of many related moth species. Therefore, it seems likely that the bombykal-cell of male silkmoths ensures sexual isolation (Daimon et al.2012). The two neurons, which are housed in long trichoid sensilla of females, however, neither detect bombykol nor bombykal but are tuned to a small number of terpenes ('terpene-cell'), or acids ('benzoic acid-cell'), respectively (Heinbockel and Kaissling1996, Priesner1979). The molecular receptive range of medium-sized trichoid sensilla is not known.

In many species of moths, males also release pheromones via hair pencils, modified abdominal scales, which are detected by trichoid sensilla on the female antenna (Hillier et al.2006), followed by female attraction or acceptance (Hillier and Vickers2004, Shorey1973, Teal1981). *Bombyx mori* males also possess hair pencils at the first abdominal segment, and despite numerous efforts male-specific odors released by these hair pencils have not yet been identified (Anderson et al.2009). Moreover, neurons of female trichoid sensilla can be activated by plant-released compounds, which facilitate the female to locate suitable host plants for their larvae (Bruce et al.2005, Shields and Hildebrand2001, Visser1986). In *Bombyx mori* larvae are oligophagous and feed mainly on leaves of mulberry trees (*Morus* spp.), while adults have no mouthparts and are therefore not able to feed.

After having detected bombykol, male silkmoths immediately start fanning their wings, and walk towards the pheromone-releasing female (Butenandt1959). Although the wings of silkmoths are well developed, the moths are not able to fly anymore, unlike their wild ancestors. Females even remain largely sessile throughout their adult stage, probably also an observed consequence of domestication. The behavioral relevance of odors detected by female sensilla trichodea is still elusive. It has been suggested that the plant-related odorant linalool might serve as an oviposition cue (Rostelien et al.2005).

Benzoic acid is not typically released by plants, and therefore might play a role in a different behavioral context, e.g. as a putative male-specific scent. Heinbockel and Kaissling (1996) demonstrated that the meconium of *B. mori* larval waste products that are excreted after eclosion, could activate the ‘benzoic acid-cell’. Nevertheless, no specific behavior could be observed in females after presenting either benzoic acid or meconium.

The present study was performed with the goal of addressing the question of which ecological relevant odorants can be detected by trichoid sensilla of female silkmoths, and what behavioral significance these odorants might have. We first collected and identified volatiles of mulberry leaves, the exclusive host plant of *B. mori*. We furthermore aimed at finding potential male-specific scents, and therefore collected odors from male and female silkmoths, male and female meconium, and of male-specific hair pencils. Then, we tested the physiological activity of these compounds using single sensillum recordings. Since we hypothesized that trichoid sensilla might be involved in oviposition or mating, we examined the influence of the female’s reproductive status on the sensitivity of their olfactory neurons. In order to investigate the ecological meaning of the identified best ligands we established a two-choice behavioral assay for female silkmoths (Y-maze).

MATERIAL AND METHODS

Animals

Pupae of the domesticated silkmoth *Bombyx mori* (hybrid strain Kinshu (Japanese) x Showa (Chinese)) were purchased from Aseptic Sericulture System Laboratory (Kyoto, Japan). Male and female pupae were kept separately at room temperature. After eclosion moths were transferred in a refrigerator at 4°C until their usage for experiments. For mating experiments a couple of silkmoths were placed in a plastic box (8 cm x 6 cm x 9 cm). Seven to thirteen hours after the copulation started, the couples were separated and the females used for experiments.

Volatile collection and Analysis

We collected the headspace of mulberry leaves, male and female silkmoths and the meconium of both sexes. Mulberry leaves or two moths at a time were placed in a 500 ml and a 100 ml Schott bottle, respectively. Via a push system a humidified air flow of 0.3 l/min was adjusted. The odor enriched air left the closed system via a connected glass tube packed with 25 mg POR-Q (VCT, USA). These odor traps were cleaned twice with methanol, chloroform, acetone, dichlormethane and hexane before usage. Headspace collections were done for 24 hours and the traps were subsequently removed and eluted with 300 µl hexane. For gas chromatography mass spectrometry (GC-MS) mulberry extracts were used pure, while odor collections of female and male silkmoths were concentrated under a gentle stream of nitrogen to 50 µl. The headspace of freshly collected meconium was collected in a 1ml glass vial for 1 hour using a grey solid phase micro extraction (SPME) fiber (50/30 µm DVB/ CAR/ PSMD, SUPELCO) and immediately analyzed via gas GC-MS. For hair pencil odor collections we removed the hair pencils of several males with forceps, and introduced them into a glass insert containing 100 µl of hexane. We collected both hair pencils of 6 to 20 males per insert. Scales from the head of the males were used as control. After the evaporation of hexane, the glass inserts containing hair pencils or control scales were analyzed by connecting the GC-MS with a thermal desorption unit (=TDU).

All extracts, compounds collected with SPME fibers and hair pencils were examined on an Agilent7890A gas chromatograph (AgilentTechnologies,CA) running in splitless mode and being connected to an Agilent 5975C mass spectrometer (electron impact mode, 70eV, ion source: 230°C, quadrupole: 150°C, mass scan range: 33–350 u). We used either a nonpolar column (HP-5 MS UI, 30 m length, 0.25 mm ID, 0.25 µm film thickness, J and W Scientific) or a polar column (HP-INNOWAX, 30 m length, 0.25 mm ID, 0.25 µm film thickness, J and W Scientific) under constant helium flow of 1.1 ml/min. The GC oven for the two columns was programmed as shown in Table 1.

Chemicals

In Table S2 all synthetic odorants tested are listed. They were commercially available and acquired from Sigma-Aldrich (http://www.sigma-aldrich.com), Chem Faces (http://www.chemfaces.com/), or BOC Sciences (https://www.bocsci.com/) in the highest available purity. The linalool enantiomers were kindly provided by Wittko Francke. Bombykal was kindly synthetized from bombykol (Pherobank, https://www.pherobank.com/) by Jerrit Weißflog. For electrophysiological experiments 6 serial dilutions of odorants were made with hexane starting from 100 µg. For behavioral tests compounds were diluted in mineral oil (0.1 µg).

Single sensillum recordings

In order to investigate the response properties of trichoid sensilla, we performed cut tip single sensillum recordings. The antenna of a one to eight day old female was cut at the base. The glass capillary of the reference electrode filled with hemolymph ringer (Kaissling1995) was introduced into the base and sealed with Vaseline. With a custom-sharpened forceps the tips of trichoid sensilla were cut. The glass capillary of the recording electrode was filled with sensillum ringer (Kaissling1995). The antenna was placed under the microscope. A PEEK tube, providing a constant, humidified clean air stream (main flow, 0.5 l/min), was directed toward the recording site with a 2 cm distance. When inserting an odor stimulus (0.4 l/min, 500 ms odor pulse) into the main air stream the main air stream switched automatically to an additional compensatory air flow (Syntech CS-55 Stimulus Controller, Kirchzarten, Germany). Using a micromanipulator the recording electrode was put over the tip of a cut trichoid sensillum. For stimulations filter papers loaded with odorants were prepared before experiments. 6 µl of each dilution were pipetted on a filter paper, which was placed in a glass pipette. The software Auto Spike32 (version 3.7) measured changes in extracellular potentials. Signals were amplified 10× (Syntech Universal AC/DC probe), sampled with 48000 Hz, and filtered (300–3 kHz with 50/60 Hz suppression). Neuronal activity was recorded 3 s before and 20 s after stimulation. Each sensillum type (T1 and T2) was recorded only once per female antenna. We analysed the action potential frequency (spikes/s) over the total recording interval using a bin width of 25 milliseconds. In T1 sensilla, two neurons could unambiguously be differentiated based on their different spike amplitudes; while the two neurons housed in T2 sensilla had similar spike amplitudes in most of the cases. By calculating the difference of the maximum frequency between 1 second before and 1 second after stimulus onset, we quantified the physiological response of the olfactory sensory neurons upon odor stimulation.

Binary-choice behavioral assay

In order to test the behavioral relevance of odor stimuli we established a binary choice assay for female silkmoths, a Y-maze (Fig. 4A, B). Each experimental arm of the Y-maze (diameter 28 mm, length 120 mm) was connected to a 100 ml glass bottle containing 1 ml of the solvent mineral oil (control arm) or 1 ml of the diluted odorant (10^{-4}, test arm). In a control experiment, both bottles contained 1 ml mineral oil. Via a valve humidified air was pulsed for 2 seconds (interval 2 s) through the glass bottles into the Y-maze at 0.3l/min. Air was pulled out through the entrance arm of the Y-maze at 0.9 l/min to ensure the odor flow through the setup. A camera recorded the moth behavior from the top. Mated females (1-4 d old) were placed in the experimental chamber 30 min before testing. Females were tested at 25 °C and a relative humidity of 70% at the end of the photophase immediately after copulation (Yamaoka and Hirao1981). A single female was placed in the Y-maze and was observed until she made her first decision. Entering one of the arms of the Y-maze with the thorax (Fig. 4 B, red line) was defined as decision of the female. The maximal recording time was 10 minutes. In order to prevent site-biased effects the position of the control and test arm was switched after each experiment. For estimating the valence of an odor we analyzed the number of responding females, i.e. females' that made a choice, and measured the latency (s) until they took their decision, and noted which arm of the Y-maze was chosen. When a female turned downwind after she started moving, her behavior was classified as aversive. Furthermore, we counted the number of females laying eggs and the number of females flapping their wings, and measured the total duration of wing flapping (s) for each female.

Analysis

All statistical analysis was done with InStat 3 and GraphPad Prism 4. For visualization of the data we used SPSS Statistics 17.0, GraphPad Prism 4 and Adobe Illustrator CS5. Differences in the amount of linalool enantiomers in the mulberry odor collection were tested with Wilcoxon matched pairs test.

Physiological response to an odor stimulus was tested subtracting the solvent response from the calculated physiological response upon odor stimulation and using a Wilcoxon rank sum test against zero. In order to compare the sensitivity of virgin and mated females, we calculated the half maximal effective concentration (EC50) for each dose response curve, and quantified significant differences with an F test.

If an odor cue had an effect on the females' choice behavior, first decisions for the odor arm and for the solvent arm would be significantly different from a 50:50 distribution (Chi square test for goodness of fit). By using a Kruskal-Wallis with post test the decision latency and the relative time females spend with wing flapping were analyzed for each odor treatment in comparison to the response toward the solvent mineral oil. Wing flapping activity was quantified in all tested

females, that is for responders observation times equaled decision latency, while for non-responders the observation time was 10 min (maximal duration of experiment). For each odor treatment differences in the number of non-responders, females showing aversive behavior, and females laying eggs were tested against the respective result of the solvent experiment (Fisher's exact test with Bonferroni-Holm correction).

RESULTS

Our first aim was to characterize the response spectrum of trichoid sensilla of female silkmoths. Therefore, we compiled a large set of odorants, including: odors that have been described to activate sensilla of female silkmoths (Anderson et al.2009, Heinbockel and Kaissling1996, Priesner1979), common floral and leaf volatiles, and odorants that were present in the headspace of mulberry leaves (Fig. S1A). We also collected volatiles from male hair pencils to identify potential male pheromones. However, there was no difference between the volatile profiles of hair pencils compared to that of 'normal' scales from the male's head (data not shown), despite the usage of the highly sensitive TDU-GC-MS. Male-specific compounds might be emitted by other structures than hair pencils, and we therefore collected volatiles of both male and female silkmoths, and found a total of 41 compounds, mainly consisting of terpenes (Fig. S1B). The only male-specific compound detected was acetophenone as it was found in the headspace of 10 out of 13 males but in none of the 10 females tested. Since it has been shown that meconium elicited electrophysiological responses in female trichoid sensilla (Heinbockel and Kaissling1996), we analyzed the volatiles released by the meconium of males and females (Fig. S1C). We encountered similar components detected in the headspace of *B. mori*, because most moths excreted meconium during the 24 hours of odor collection, but we additionally found, nitrogen- and sulfur-containing components. None of these compounds were present in only one sex, and we thus included 9 of the odors identified in the headspace of silkmoths and their meconium in our test set of 77 odorants. As these odors might play a biological role for female silkmoths, and might therefore be detected by female trichoid sensilla. Notably, benzoic acid, or other acids, could not be found in any of the odor collections.

Single sensillum recordings revealed two functional types of female trichoid sensilla

Based on their physiological characteristics, we identified two types of trichoid sensilla. Type 1 (T1) contained two neurons, the "terpene cell" (T1A), with large spike amplitudes and very scarce spontaneous activity, and the "benzoic acid cell" (T1B), with smaller spike amplitudes and a spontaneous activity of 17 Hz ± 9.8 (mean ± SD, Fig. 2A, (Heinbockel and Kaissling1996, Priesner1979). Despite previous reports of T1 sensilla being finely tuned to linalool, α-terpineol and benzoic acid, our results demonstrate that the two neurons of T1 sensilla are more broadly tuned to odorants (Fig. 2A, B). In addition to linalool and α-terpineol (Boeckh et al.1965) cis-

jasmone led to a strong activation of an average greater than 105 Hz in the T1A neuron (Fig. 2A). Since (+)-Linalool is the enantiomer of racemic linalool that was almost exclusively emitted by mulberry leaves (96%, Fig.S1A), we included (+)-linalool and (-)-linalool in our odor set, and found that (+)-linalool elicited a stronger response of the T1A neuron than (−)-linalool ($p = 0.004$, $n = 8$, Wilcoxon matched-pairs test). Additionally 12 odorants, being mainly aromatic compounds and terpenes, and notably the male-specific compound acetophenone, increased the spiking frequency of T1A to a lesser degree (average responses <50 Hz). The "benzoic acid cell" of T1 sensilla was activated by most of the acids tested (Fig. 2B). The best ligands for the T1B neuron beside benzoic acid were benzaldehyde, isovaleric acid and pentanoic acid (average responses >120 Hz). Interestingly, we could not confirm that the meconium of silkmoths activates T1B neurons (Heinbockel and Kaissling1996). In total 22 compounds activated T1B, whereas two volatiles (p-cresol, indole), led to an inhibition of the firing rate in the same cell.

Besides this already known trichoid sensillum type, we discovered a new type (T2) housing also two neurons. Different to T1 neurons, which could easily be discriminated by their different spike amplitudes, both T2 neurons exhibited often very similar spike amplitudes. Based on this observation we suggest that T2 sensilla belong to the medium-sized trichoid sensilla, housing two morphological similar neurons (Steinbrecht1970). Due to this similarity we could separate them only in a few cases (example in Fig. 2C). Therefore, we analyzed the responses of both neurons together ('T2AB'). Moreover, T2AB neurons fired with an average spontaneous firing rate of 2 Hz ± 3.8, which was a much lower frequency than T1B neurons (17 Hz), and higher than the scarce spontaneous activity observed in T1A neurons. Apart from these different characteristics, the receptive range of T2AB neurons was generally similar to that of T1A neurons (Fig. 2C). Twelve odorants, mainly terpenes and aromatics, elicited a significant response, with cis-jasmone, α-terpineol and methyl salicylate as best ligands (average responses > 110 Hz). Notably, cis-jasmone, being present in a very low amount in mulberry leaves compared to all analyzed chemicals (Fig. S1A), is a highly attractive olfactory cue for the larvae of *B. mori* (Tanaka et al.2009). In contrast to T1 neurons, however, T2 neurons were activated by the mulberry odor, and did not respond to acids as T1B neurons did. A principal component analysis using the response profiles of T1A, T1B and T2AB neurons (Fig. 2) confirmed the presence of the sensillum and neuron types that we identified based on their physiological properties like spike amplitude and spontaneous activity (Fig. S2). Hence, we could describe the molecular receptive range of a second type of trichoid sensilla in female silkmoths, and could confirm and expand the response profile of the already known trichoid sensillum type (Boeckh et al.1965, Priesner1979).

Mating enhanced the sensitivity of type 2 trichoid sensilla

Since we hypothesized that trichoid sensilla might be involved in either host plant detection or in pheromone communication, we compared the sensitivity of neurons housed in trichoid sensilla in

virgin females, with the respective sensitivity of neurons in mated females. If trichoid sensilla would play a role in finding an appropriate oviposition site, we would expect an increased sensitivity in mated females. On the other hand, if trichoid sensilla would have a function in precopulation behavior, virgin females' sensilla might be especially sensitive. We performed dose-response experiments across five orders of magnitude (60 ng to 600 µg, examples in Fig. 3A, C, E) using three activating odorants for T1A, T1B, and T2AB neurons, and also included one of the two compounds that inhibited the spontaneous activity of T1B neurons. As a measure for a potential change in sensitivity we calculated the odor concentration that elicited the half maximum response of a neuron (EC50 value), and compared these values between virgin and mated females (Table 1). T1 neurons generally showed similar dose-response curves in virgin and mated female moths with two exceptions: stimulations with (+)-linalool revealed a higher sensitivity of T1A neurons towards this compound in mated females (Fig. 3B, Table 1). T1B neurons of virgin females, in contrast, were more sensitive to isovaleric acid, than T1B neurons of mated females (Fig. 3D upper panel, Table 1).

The inhibitory effect of indole on the spontaneous activity of T1B was similar in virgin and mated females (Fig. 3D lower panel, Table 1). However, at the highest concentration of indole, T1B neurons of virgin females were inhibited for 3.1 s ± 1.4 (mean +/-SEM), whereas in mated females this inhibition lasted only 1.6 s ± 1.

In contrast to T1 neurons, the reproductive status of a female had a huge impact on the sensitivity of T2AB neurons (Fig. 3F). Stimulations with cis-jasmone, methyl salicylate, and (+)-linalool revealed that these neurons had 8 to 16-fold lower EC50 values in mated females than in virgin females (Fig. 3F, Table 1). Thus, we could show that mating increased the sensitivity of T2AB neurons drastically, indicating that these neurons rather play a role in the detection of host plants than in pheromone communication. For T1 neurons, however, no clear conclusions can be drawn, as the sensitivity of T1A neurons was higher in mated females, while the sensitivity of T1B neurons was higher in virgin females. Moreover, the opposing impact of the reproductive status on T1 neurons (3 to 4-fold change in EC50 values) was low compared to T2AB neurons.

Odor-guided behavior of female silkmoths

In order to investigate the behavioral relevance of odorants activating trichoid sensilla, we performed a binary-choice assay with mated females (Fig. 4A, B). We also included indole in our behavioral experiments as this odorant inhibited T1B neurons. We presented (+)-linalool (T1A), isovaleric acid (T1B), indole (T1B), or cis-jasmone (T2AB) in one of the arms of a Y-maze, while the solvent mineral oil was present in the other arm. In a control experiment, we used mineral oil in both arms to test for a potential bias in the assay.

In all test conditions, a similar number of females (58-77% of 60 animals tested in each experiment) decided for one of the arms within 10 minutes (Fig. 4C, left). However, only in the

experiment with cis-jasmone, the test arm attracted more females than the control arm (Fig. 4C, right). In other experiments including the control experiment, we observed a random distribution of females. Independent of the stimulus, responders required about 220 s until they made their decision (Fig. 4D).

By recording females during the experiments we quantified further odor-driven behaviors of all 60 animals tested. In insects, aversion is often observed as downwind movement (e.g. Steck et al.2012), and we therefore counted the number of females that turned by 180 degrees after they were placed in the Y-maze (Fig. 4E). In the control experiment, 20 % of the moths turned downwind (dashed line in Fig. 4E). Similar results were obtained when presenting cis-jasmone or (+)-linalool. Ligands affecting T1B, however, elicited more than twice as often this aversive behavior.

Silkmoths were mated immediately before the behavioral experiments. Therefore, they were highly motivated to lay eggs. Even in the control experiment, 65 % of all tested females started to oviposit (Fig. 4F, dashed line). While indole and cis-jasmone did not affect oviposition, almost all tested females laid eggs when perceiving (+)-linalool. Interestingly, also the aversive odor isovaleric acid raised the number of ovipositing females.

Although *B. mori* is not able to fly, male silkmoths show a characteristic wing flapping behavior in response to the female sex pheromone (Butenandt1959, Schneider1956). In female silkmoths, a similar behavior was described towards a terpene that activates T1A neurons (Priesner1979) but no response was observed to benzoic acid (T1B) (Heinbockel and Kaissling1996). Nevertheless, wing flapping behavior in females was not quantified so far. Thus, we counted the number of wing flapping females in each experiment (Fig. 4G), and calculated the duration of this behavior (Fig. 4H). In comparison to the control situation, the aversive odorants isovaleric acid and indole, as well as the attractive odorant cis-jasmone significantly increased the number of wing flapping females. Moreover, during the experiment with cis-jasmone, females spent longer time with wing flapping compared to the control experiment (Fig. 4H).

From these experiments (+)-linalool, the best ligand for T1A neurons, could be seen to increase oviposition in mated females.. Enhanced wing flapping responses were observed both in the presence of the aversive volatiles isovaleric acid and indole (T1B), and the attractive compound cis-jasmone (T2AB).

DISCUSSION

In our study we characterized two types of trichoid sensilla on the antenna of female *B. mori* belonging to long-sized (T1) and medium-sized (T2) trichoid sensilla. Thereby, we found a new, so far functionally undescribed trichoid sensillum type. We showed that this sensilllum type was also highly sensitive towards host plant-released volatiles. After mating, the sensitivity toward

plant-related compounds was even more enhanced. With our findings we underlined the importance of plant volatiles for female moths, since searching suitable oviposition sites in order to ensure the availability of food for their larvae is one of the major tasks of a female (Bruce et al.2005). Furthermore, we demonstrated that volatiles, which were detected by trichoid sensilla, were behaviorally relevant in female *B. mori* by causing specific odor-dependent behaviors such as wing flapping.

We characterized trichoid sensilla of female *B. mori*. In order to record from single sensilla we cut the sensillum and could therefore not assign the length of a sensillum to a functional type. In electrophysiological recordings thick dendrites of a neuron result in larger spike amplitudes compared to thinner dendrites (Pettersen and Einevoll2008). While we were able to differentiate T1A and T1B neurons unambiguously, recordings of T2A and T2B neurons revealed similar spike amplitudes. According to Steinbrecht (1973), neurons of long-sized trichoid sensilla are reported to be morphological different, but not neurons of medium-sized trichoid sensilla; our results suggest that T1 belongs to the first sensillum type, while T2 belongs to the second one.

Long-sized trichoid sensilla comprise 38 % of all sensilla of the female antenna (Steinbrecht1970) but are completely lacking in other lepidopteran species (Boeckh et al.1960, Sanes and Hildebrand1976). In line with previous findings (Heinbockel and Kaissling1996, Kaissling and Priesner1970), we observed only one functional type of T1 sensilla, similar to the only pheromone-responsive trichoid sensillum type in males. This underlines the importance of T1 in females and leads to speculations concerning a role in pheromone detection. Furthermore, our results confirm electrophysiological observations made in T1 sensilla (Boeckh et al.1965, Heinbockel and Kaissling1996, Priesner1979) by demonstrating that neurons of this sensillum type are strongly activated by racemic linalool, α-terpineol (T1A), benzoic acid and benzaldehyde (T1B). So far, it is considered that long-sized trichoid sensilla are involved in host detection (Heinbockel and Kaissling1996). In addition to previous studies, we extended the electrophysiological investigations. We examined the receptive range of T1A and T1B neurons by using a large odor panel and demonstrate that both neurons respond to a broad range of compounds. Interestingly, the A-neuron of long-sized T1 sensilla was weakly activated by the male-specific odorant acetophenone, which is known to be a hair pencil compound of the African monarch *Danaus chrysippus* (Schulz and Vane-Wright1993). Another compound, which activated T1A neurons weakly and which is also known as a pheromone in butterflies (Francke1989, Mann et al.2017, Schulz and Vane-Wright1993) and ants (Rocca et al.1983), was the meconium-emitted compound dihydroactinidiolide. Nevertheless, the mulberry compound (+)-linalool, was the best ligand of T1A neurons, and elicited a 9 fold stronger response than acetophenone. In contrast, linalool is also known as a male-specific hair pencil pheromone in *Trichoplusia ni* (Heath et al.1992). Moreover, in males the second neuron of long trichoid sensilla detects a putative minor pheromone component of *B. mori* (Kaissling and Priesner1970, Kaissling et al.1978) and is therefore also involved in pheromone detection. T1B neurons of female silkmoths responded mainly to acids and benzaldehyde. The role of acid sensing in

female *B. mori* is still unknown. We could not identify acids in our odor collections; however components like benzoic acid and benzaldehyde are known to be present in hair pencils of noctuid moth species (Birch and Poppy1990). Although we could not identify male-specific compounds in the hair pencils, a role of T1 sensilla in pheromone communication cannot be excluded. The lack of such volatiles might be an effect of domestication. Investigating the hair pencils of the wild ancestor of B. mori, *Bombyx mandarina*, would help to understand the role of these structures.

Besides the trichoid sensillum type 1, we characterized a second so far functionally not described type (T2) responding exclusively to plant volatiles. T2AB neurons were most sensitive to cis-jasmone, as only 148 ng of this compound were necessary to elicit the half maximum response in mated females (Table 1, Fig. 3F). This was about 4 times less than methylsalicylate and 6 times less than (+)-linalool. Cis-jasmone is also a highly attractive olfactory cue for larvae of *B. mori* (Tanaka et al.2009). In this study, the mulberry leaf odor attracts the larvae similarly as only 0.3 ng of cis-jasmone. Moreover, the sensitivity of T2 sensilla to plant volatiles like cis-jasmone, but not T1 was drastically increased by mating suggesting a key role of T2 sensilla in finding an appropriate oviposition site. A mating-dependent increase in the sensitivity to host plant volatiles was also observed in the behavioral consequence of other lepidopteran species (Landolt1989, Masante-Roca et al.2007, Mechaber et al.2002). As shown in many moth species, more than one physiological type of medium-sized trichoid sensilla exist (e.g., in *Manduca sexta*, (Ghaninia et al.2014, Shields and Hildebrand2000); in *Heliothis virescens*, (Hillier et al.2006). According to the previous findings and in line with the study of Steinbrecht (1970), in *B. mori* at least one additional functional type of medium-sized trichoid sensilla can be expected beside the characterized T2.

In order to reveal the behavioral relevance of odorants detected by trichoid sensilla, we tested female silkmoths in a Y-maze. To our knowledge, our study is the first that dissected and quantified odor-guided behavior in female *B. mori*. Our results confirmed previous findings that oviposition in female *B. mori* is not enhanced in the presence of cis-jasmone (Damodaram et al.2014). But rather we demonstrated that, similar to behavior observed in larvae (Tanaka et al.2009), cis-jasmone is an attractant, since females showed upwind movement and chose the test-arm of the Y-maze containing cis-jasmone. Furthermore, females were not only attracted by the mulberry volatile cis-jasmone, but this compound also elicited a long lasting wing flapping behavior in the females, similar to a male pheromone response. Together, these observations underlines the important role of cis-jasmone, which is suggested as a specific cue of mulberry plants (Tanaka et al.2009), not only in larvae, but also for adult female silkmoths. Tanaka and colleagues (2009) investigated the receptor involved in larval attraction due to stimulation of cis-jasmone, where they could demonstrate that cis-jasmone specifically activates the receptor BmOr56. The receptor is shown to be also expressed in adults, and thus, the question arises whether BmOR56 is expressed in medium-sized trichoid sensilla of the adults and mediate the cis-jasmone attraction. Suggested future experiments include the visualization of the receptor by

in situ hybridization and knock-out experiments of BmOR56 in adult moths in order to address the possible role of BmOR56 in the detection of cis-jasmone.

Short wing flapping was also observed toward isovaleric acid and indole, but instead of demonstrating attractive behavior, females seemed to try to avoid such volatiles by turning downwind. Wing flapping behavior in silkmoths is considered to reflect the initiation of flight by being attracted to e.g. a pheromone source in males (Butenandt1959). Here, we demonstrated that wing flapping behavior is associated with either attraction or aversion. Acids are known as fermentation products and could serve as negative cues, which would be strengthened by the fact that in our behavioral assay female silkmoths responded with aversion toward isovaleric acid. Measuring the behavioral response of females toward a blend consisting of host plant cues and acids in order to mimic fermenting host plants might help to understand the ecological relevance of acids. Indole, the second odorant eliciting aversion in our behavioral assay, inhibited T1B neurons in females. The role of peripheral inhibitory effects is not yet clearly understood, and has been (Cao et al.2017) suggested as a means to increase efficiency of odor coding (Cao and colleagues, 2017). Besides attraction and aversion behavior, we observed compounds serving as oviposition cues such as (+)-linalool. A high number of females started to oviposit during testing, but almost all females tested laid eggs in response to (+)-linalool, where this compound has also been suggested to mediate egg laying in *Manduca sexta* (Reisenman et al.2010). Interestingly, the number of ovipositing females was also increased in the presence of isovaleric acid. Acids like acetic acid (Goegues et al.2011) or octanoic acid (Elmaci and Altug2002) are contained in mulberry fruits, which might explain the increase of oviposition towards an acid. Further investigations are necessary in order to understand the role of acids in female silkmoths.

In several insect species it has been demonstrated that odor-induced activity at the sensory level correlate with the behavior (Bisch-Knaden et al.2018, Guerrieri et al.2005, Knaden et al.2012, Kuebler et al.2012). We cannot exclude the contribution of other sensillum types to the observed behavioral performance for the tested compounds, since odor valence is determined by odor processing within the neuronal network. Coeloconic sensilla detect certain compounds, which are contained in mulberry leaves but have not been shown to detect linalool (Pophof1997). Mulberry odors are detected by basiconic sensilla in addition (Priesner1979), whereas racemic linalool inhibits the neurons being housed in this sensillum type (Ziesmann et al.2000). Thus, detection of linalool by more than a single sensillum type suggests odor processing within the neuronal network. All three olfactory sensillum types on the female antenna seem to be involved in the detection of host volatiles of mulberry leaves. The contribution of other sensillum types, besides trichoids to the detection of cis-jasmone is so far unknown.

Taken together, our study underlines the importance of host plant volatile detection for female silkmoths in order to find suitable oviposition sites. We found olfactory cues for attraction, aversion and oviposition. Moreover, the results presented here suggest that medium-sized trichoid sensilla being highly sensitive to cis-jasmone are involved in host search. Despite efforts to determine the role of long-sized trichoid sensilla in odor detection and in mediating behavioral

responses, their specific biological function is still puzzling. This sensillum type might be involved in host detection as well, and additionally a role in the detection of pheromones cannot be excluded.

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SUPPLEMENTAL INFORMATIONS

Supplemental information includes two figures (Fig. S1 and Fig. S2) and two tables (Table S1 and table S2)

FIGURE LEGENDS

Figure 1: Shape of the antenna of silkmoths. Similar antennae of male (left panel) and female (right panel) *B. mori*.

Figure 2: Receptive range of olfactory sensory neurons housed in trichoid sensilla of virgin female *B. mori*. Upper panel shows representative traces of electrophysiological recordings from type 1 **(A, B)** and type 2 **(C)** trichoid sensilla of the same animal with indicated odor stimuli (60 µg on filter paper). Solvent control was hexane or acetone (benzoic acid). Grey bars indicate odor stimulation. Bars in the lower panel show average (±SEM) maximum spike frequencies of T1A **(A**, dark green, n=9**)**, T1B **(B**, light green, n=9**)** and T2AB neurons **(C**, blue, n=8**)** after stimulation with monomolecular compounds, mulberry extract (6 µl), freshly collected meconium (6 µl), and solvents (hexane, acetone). Based on Knudsen et al. (2006), odorants were categorized to the chemical class they belong to. Filled bars indicate a significant neuronal activation ($p < 0.05$, Wilcoxon rank sum test).

Figure 3: Mating enhanced the sensitivity of neurons housed in T2 trichoid sensilla. Representative traces of recordings from T1A (dark green) **(A)**, T1B (light green) **(C)** and T2AB neurons (blue) **(E)** of a mated female silkmoths with indicated odor stimuli in three different concentrations (0.6 µg, 6 µg und 60 µg). Recordings of both sensillum types belong to the same animal. Solvent control was hexane. Grey bars indicate 500 ms of odor representation. **(B, D, F)** Quantified dose response curves of selected ligands for each neuron and sensillum type in virgin (solid line) and mated female silkmoths (dashed line). Circles represent the average activation (spikes/s) upon odor stimulation (±SEM). Grey circles represent average solvent responses. Activation of a neuron was quantified by subtracting the net response towards solvent from the delta maximum frequency upon odor stimulation. Filled circles indicate significant differences from zero, i.e. odor responses that were different from the solvent response ($p < 0.05$, Wilcoxon rank sum test). Mating-dependent significant differences (asterisks) in sensitivity to odor stimulation were calculated by comparing the half maximal effective concentrations (EC50s) of virgin and mated females (see Table 1, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ F test). Numbers in brackets in the legend represent sample size.

Figure 4: Odor-guided behavior of mated female silkmoths in a Y-maze. (A) Schematic of the behavioral choice assay (Y-maze). An odorant (green, 1 µg) and the solvent mineral oil (grey) were pipetted into each of two glass bottles. The headspace accumulating in the bottles was pulsed into the arms of the Y-maze via a valve (2 s stimulus, 2 s clean air) with an airflow of 0.3 l/min. Position of bottles was switched after each tested moth. Odorants and solvent were

pulled out through the entrance arm of the Y-maze with an air flow of 0.9 l/min. **(B)** Picture of a female silkmoth during an experiment. Red dotted line represents the entrance of each arm of the Y-maze. **(C) – (H)** summarize the results of behavioral tests. Colors represent responses toward mineral oil (grey), experiments with the best T1A ligand (+)-linalool (dark green), with T1B tuned odorants isovaleric acid and indole (light green) and with the best ligand for T2AB, cis-jasmone (blue). Significant differences are shown by filled bars (C, E, F, G) or filled boxes (D, H), and with asterisks. **(C)** Decision of responders (left panel) either for the control (grey) or the test arm (colored). Numbers indicate the number of responder out of 60 females tested. The right panel shows the number of non-responder that was similar in each treatment ($p < 0.05$, Fisher's exact test with Bonferroni-Holm correction). Asterisk indicates a significant difference ($p = 0.039$, chi square for goodness of fit) from a 50:50 distribution (dashed lines). **(D)** Time required by female moths to decide for one arm of the Y-maze was similar for all treatments ($p > 0.05$, Kruskal-Wallis test). Black lines represent the median; boxes represent the interquartile range (25 % - 75 % quartile); whiskers represent minimum and maximum of the data. **(E)** Percentage of females turning downwind when placed in the Y-maze (N = 60; * $p < 0.05$, Fisher's exact test with Bonferroni-Holm correction). Dashed line represents the percentage of downwind turning females in the control experiment. **(F)** Percentage of females that started to oviposit during experiments (N = 60; * $p < 0.05$, ** $p < 0.01$, Fisher's exact test with Bonferroni-Holm correction). Dashed line represents the percentage of ovipositing females in the control experiment. **(G)** Percentage of females that showed wing flapping during experiments (N = 60; * $p < 0.05$, *** $p < 0.001$, Fisher's exact test with Bonferroni-Holm correction). Dashed line represents the percentage of wing flapping females in the control experiment. **(H)** Relative time females of **(G)** spent with wing flapping (N = 50; * $p < 0.05$, Kruskal-Wallis with post test against the control). Black lines represent the median; boxes represent the interquartile range; whiskers represent data within 1.5-fold distance of the interquartile range; black circles indicate outliers.

Figure S1: Odor collection. Data were normalized based on the highest peak. Lower panel shows the abundance of the compounds in a representative GC-MS trace. **(A)** Amount of mulberry leaf volatiles in headspace odor collections using SuperQ filters (upper panel, N = 10). Numbers highlight identified compounds, which were shown to be physiological active as demonstrated in Fig. 2. Orange box in the left panel indicates racemic linalool, which was analyzed regarding the ratio of its enantiomers in the insert to the right. When using a chiral column in the GC-MS (+)-linalool was quantified to be the most abundant enantiomer in mulberry leafs compared to (-)-linalool ($p = 0.008$, Wilcoxon matched pairs test). The median in the box plots is represented by white lines (left panel) and by a black line (right panel). The interquartile range is shown as boxes, whiskers represent data within 1.5-fold distance of the interquartile range and black circles indicate outliers (left panel). **(B)** The upper panel shows the amount of body components found in the headspace collections (SuperQ) of female (red, N = 10)

and male silkmoths (grey, N = 13). Notably, the only male-specific compound, we identified, was acetophenone (No. 4). Numbers highlight compounds that were used in single sensillum recordings. **(C)** Amount of chemical compounds found in SPME headspace collections of female (red, N = 9) and male meconium (grey, N = 8). Numbers highlight compounds that were used in single sensillum recordings. Black lines represent the median. The 50% quartile is shown as boxes, whiskers represent data within 1.5-fold distance of the 50% quartile and black circles indicate outliers.

Figure S2: Neuronal response profiles belong to two different neuron and one sensillum type. Principle component analysis (PCA) based on the electrophysiological recording data shown in figure 2. The first two principal components explained 65% of variance. Dark green indicates data of T1A neurons (N = 9), light green of T1B neurons (N = 8) and blue represent data of T2 sensilla (N = 8). Circles show 95% confidence intervals. All three groups were significantly different from each other ($p \leq 0.0012$, ANOSIM based on Euclidean distances, Bonferroni correction).

TABLES AND FIGURES

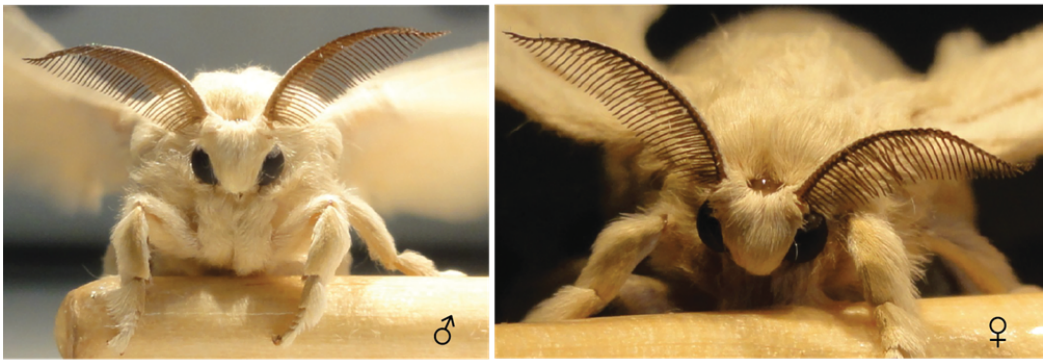
Table 1: Influence of the reproductive status on neuronal sensitivity. EC50 values [µg] of dose response curves obtained from neurons housed in trichoid sensilla of virgin and mated female silkmoths. The fitting was based on a sigmoidal dose-response curve (see Fig. 3).

OSN	Odorant	EC50 virgin females [µg]	EC50 mated females [µg]	p-value
T1A	**(+)-linalool**	**2.912**	**1.002**	**0.01***
	cis-jasmone	9.797	7.890	0.796
	methyl salicylate	7.844	20.160	0.587
T1B	**isovaleric acid**	**7.839**	**28.750**	**0.018***
	benzoic acid	4.059	8.400	0.166
	benzaldehyde	5.743	6.546	0.847
	indole	10.040	10.160	0.647
T2AB	**cis-jasmone**	**1.782**	**0.148**	**0.000*****
	methyl salicylate	**3.871**	**0.513**	**0.002****
	(+)-linalool	**13.000**	**0.825**	**0.000*****

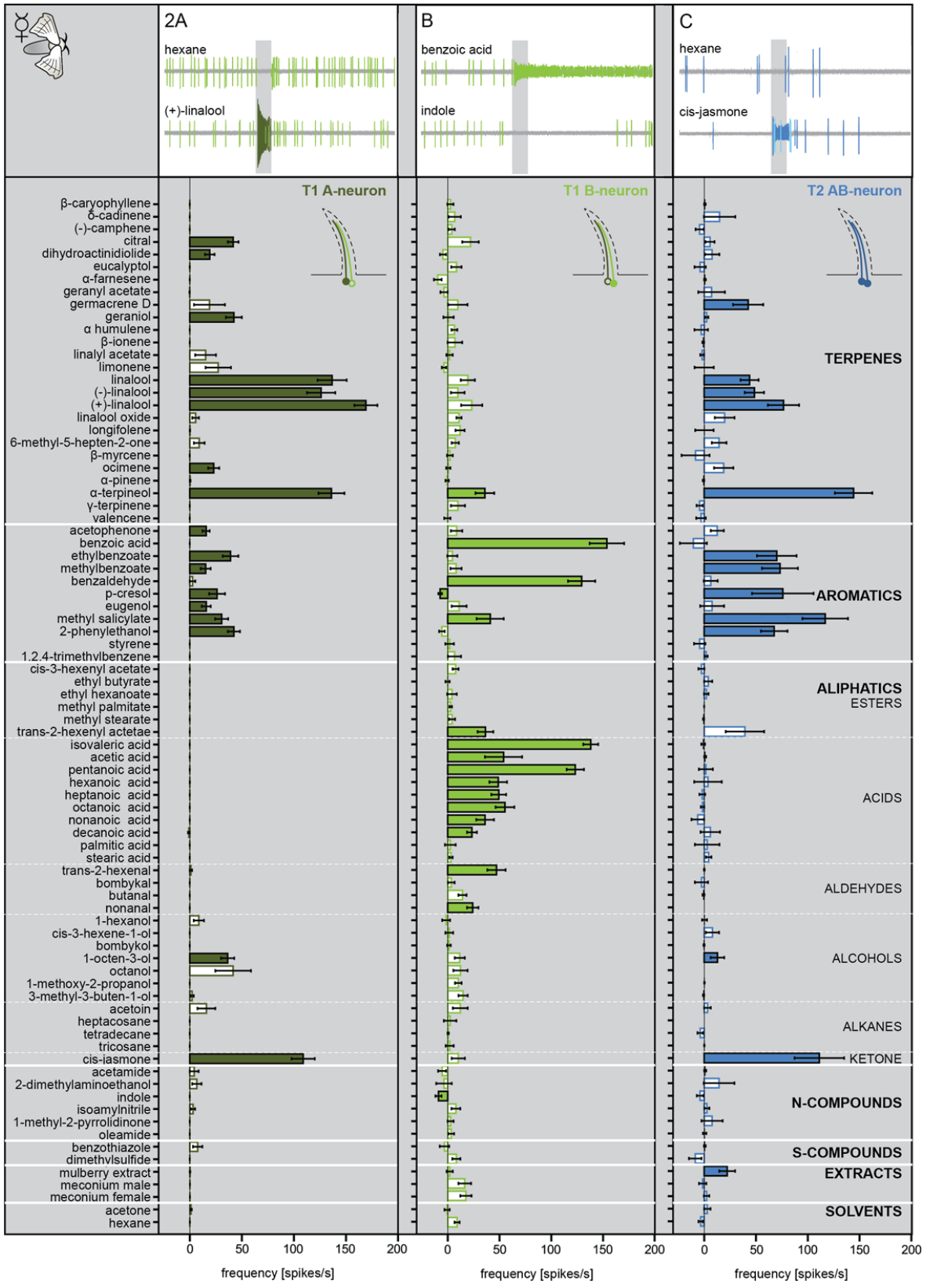
Asterisks indicate significant differences between virgin and mated females (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, F test).

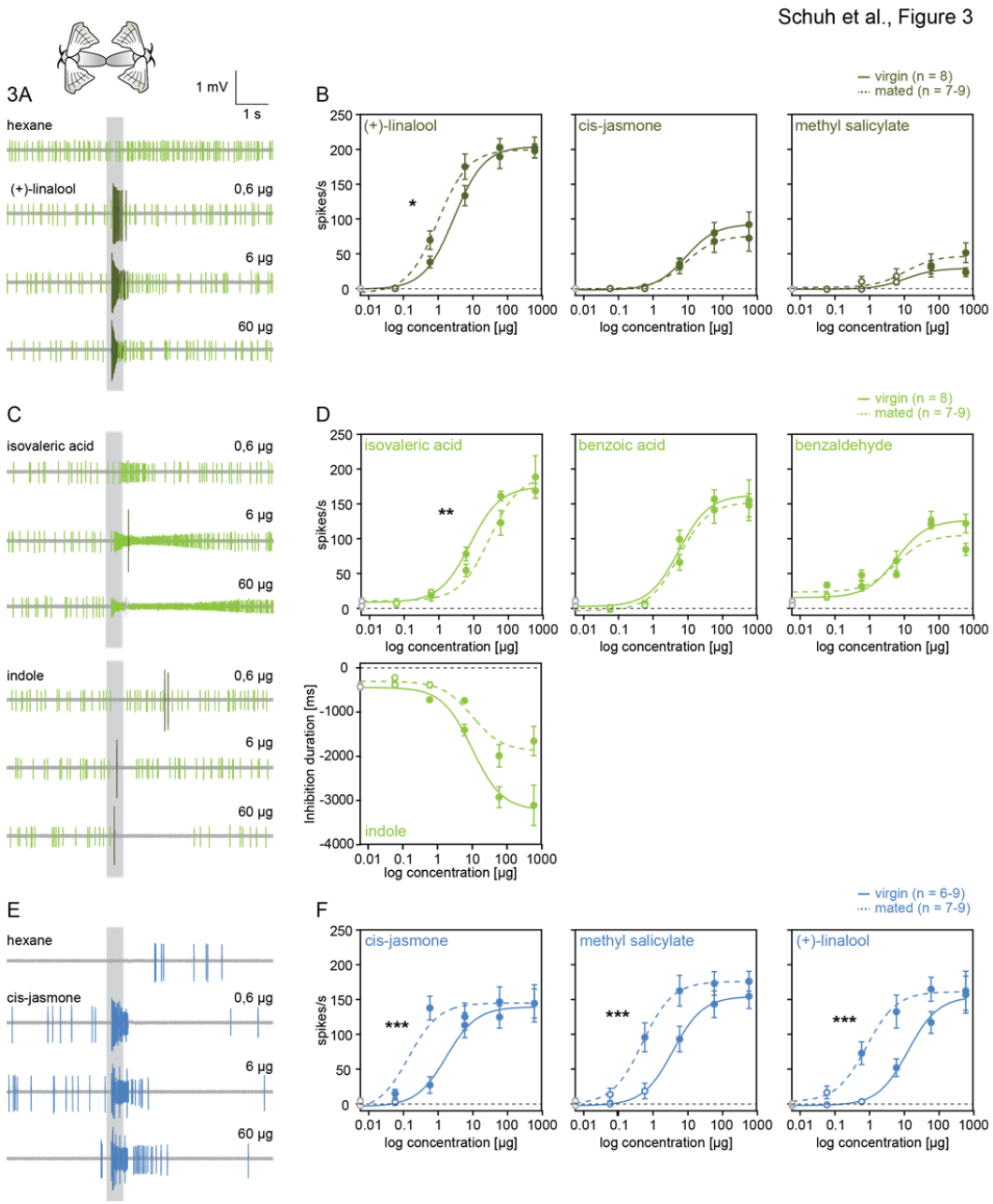
Schuh et al., Figure 1

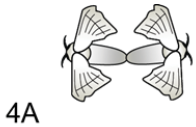
1A



Schuh et al., Figure 2

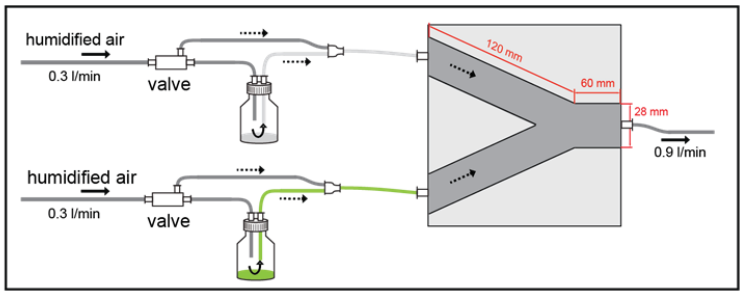



Schuh et al., Figure 3
3A
1 mV
1 s
hexane
(+)-linalool
0,6 µg
6 µg
60 µg
B
virgin (n = 8)
mated (n = 7-9)
(+)-linalool
cis-jasmone
methyl salicylate
spikes/s
log concentration [µg]
C
isovaleric acid
indole
D
isovaleric acid
benzoic acid
benzaldehyde
Inhibition duration [ms]
E
cis-jasmone
F
virgin (n = 6-9)
mated (n = 7-9)



Schuh et al., Figure 4

4A



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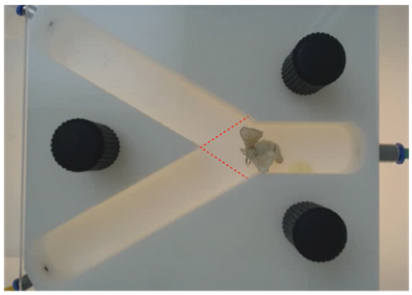

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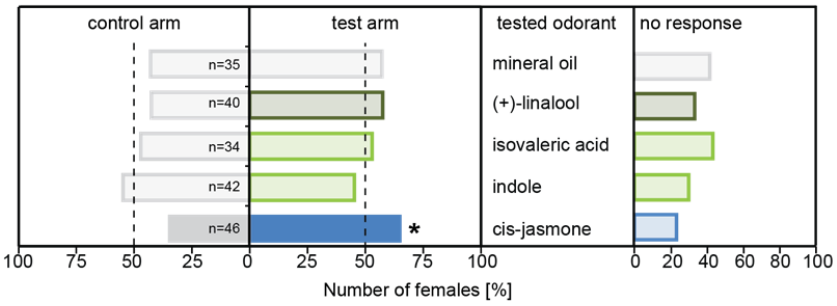

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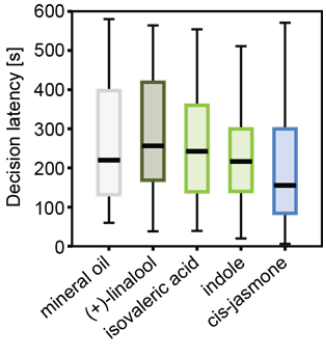
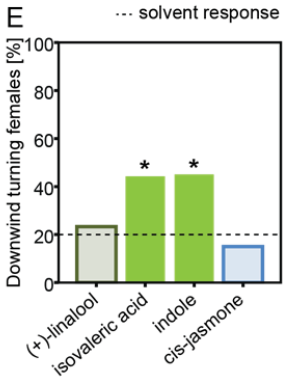


E



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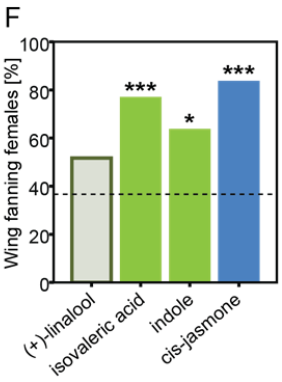

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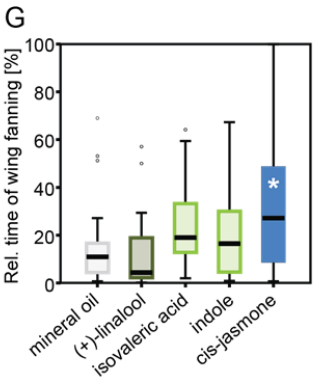


H

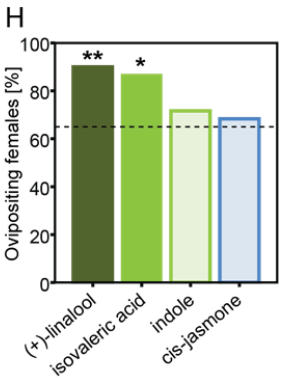


Table S1*:* Program of the GC-MS oven for all three column types. Shown is the starting temperature, the holding time at this temperature, the increase of temperature up to a maximum temperature and the holding time for the maximum temperature.

column type	Tstart [°C]	holding time Tstart [min]	Tinccrease [°C/min]	Tmax [°C]	holding time Tmax [min]
INNOWAX	40	2	15	260	10
HP5	40	3	10	280	10
Chiral	35	5	T1 = 3 T2 = 20	T1 = 120 T2 = 200	5
HP5 (TDU-GC-MS)	40	3	15	280	10

Samples being analyzed with a chiral column were heated in two phases: T1 and T2. While in phase 1 the temperature increased 3°C/min to 120°C, in phase 2 the increase changed to 20°C/min up to a maximum temperature of 200°C.

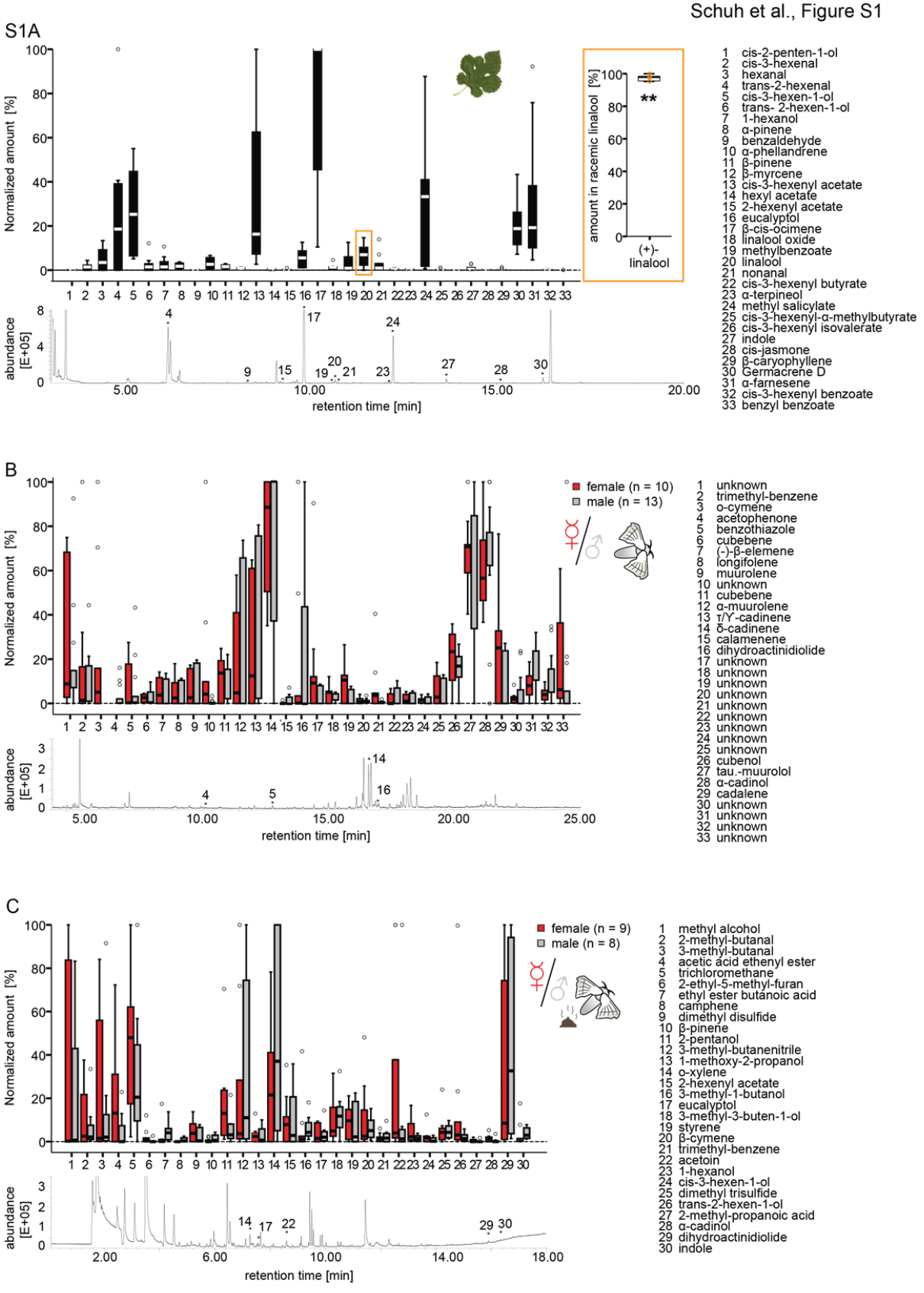
Table S2: List of selected chemicals. Chemicals are listed including CAS number and the chemical class they belong to according to (Knudsen et al.2006). Asterisks indicate components, which were identified in one of our odor collections.

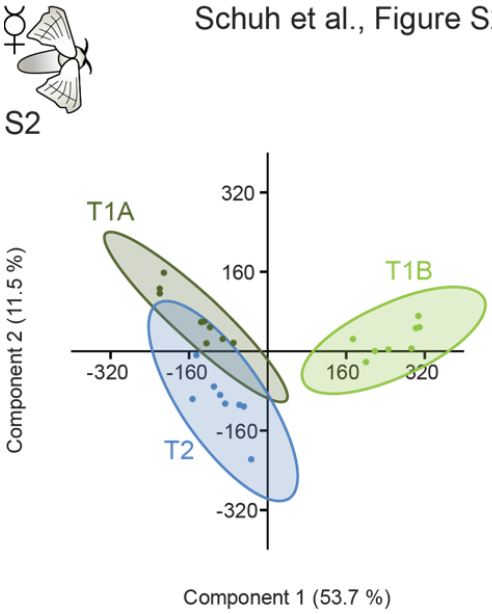
Chemical compound	Chemical Class	CAS
β-caryophyllene*	Terpene	87-44-5
δ-cadinene*	Terpene	483-76-1
(-)-camphene*	Terpene	5794-04-7
citral	Terpene	5392-40-5
dihydroactinidiolide*	Terpene	17092-92-1
eucalyptol (1,4-cineole) *	Terpene	470-82-6
α-farnesene*	Terpene	4602-84-0
geranyl acetate	Terpene	105-87-3
germacrene D*	Terpene	37839-63-7
geraniol	Terpene	106-24-1
α-humulene	Terpene	6753-98-6
β-ionene	Terpene	79-77-6
linalyl acetate	Terpene	115-95-7
limonene*	Terpene	138-86-3
linalool*	Terpene	78-70-6
(-)-linalool*	Terpene	126-91-06
(+)-linalool*	Terpene	126-90-9
linalool oxide*	Terpene	60047-17-8
longifolene*	Terpene	475-20-7
6-methyl-5-hepten-2-one	Terpene	110-93-0
β-myrcene*	Terpene	123-35-3

ocimene*	Terpene	3338-55-4
α-pinene*	Terpene	80-56-8
α-terpineol*	Terpene	98-55-5
γ-terpinene	Terpene	99-85-4
valencene	Terpene	4630-07-3
acetophenone*	Aromatic	98-86-2
benzoic acid	Aromatic	65-85-0
benzaldehyde*	Aromatic	100-52-7
p-cresol	Aromatic	106-44-5
ethylbenzoate	Aromatic	93-89-0
eugenol	Aromatic	97-53-0
methylbenzoate*	Aromatic	93-58-3
methyl salicylate*	Aromatic	119-36-8
2-phenylethanol	Aromatic	60-12-8
styrene*	Aromatic	100-42-5
1,2,4-trimethylbenzene*	Aromatic	95-63-6
cis-3-hexenyl acetate*	aliphatic, ester	3681-71-8
ethyl butyrate	aliphatic, ester	105-54-4
ethyl hexanoate	aliphatic, ester	123-66-0
methyl palmitate	aliphatic, ester	112-39-0
methyl stearate	aliphatic, ester	112-61-8
trans-2-hexenyl actetae*	aliphatic, ester	2497-18-9
3-methylbutanoic acid	aliphatic, acid	503-74-2
acetic acid	aliphatic, acid	64-19-7
decanoic acid	aliphatic, acid	334-48-5
heptanoic acid	aliphatic, acid	111-14-8
hexadecanoic acid	aliphatic, acid	57-10-3
hexanoic acid	aliphatic, acid	142-62-1
nonanoic acid	aliphatic, acid	112-05-0
octadecanoic acid	aliphatic, acid	57-11-4
octanoic acid	aliphatic, acid	124-07-2
pentanoic acid	aliphatic, acid	109-52-4
bombykal	aliphatic, aldehyde	63024-98-6
butanal	aliphatic, aldehyde	123-72-8
nonanal*	aliphatic, aldehyde	124-19-6
trans-2-hexenal*	aliphatic, aldehyde	6728-26-3
bombykol	aliphatic, alcohol	765-17-3
cis-3-hexene-1-ol*	aliphatic, alcohol	928-96-1
1-hexanol*	aliphatic, alcohol	111-27-3
3-methyl-3-buten-1-ol*	aliphatic, alcohol	763-32-6
1-methoxy-2-propanol*	aliphatic, alcohol	107-98-2
1-octen-3-ol	aliphatic, alcohol	3391-86-4
octanol	aliphatic, alcohol	111-87-5
heptacosane*	aliphatic, alkane	593-49-7

tetradecane*	aliphatic, alkane	629-59-4
tricosane*	aliphatic, alkane	638-67-5
cis-jasmone*	aliphatic, ketone	488-10-8
acetamide	nitrogen-containing compound	60-35-5
2-dimethylaminoethanol	nitrogen-containing compound	108-01-0
indole*	nitrogen-containing compound	120-72-9
isoamylnitrile	nitrogen-containing compound	625-28-5
1-methyl-2-pyrrolidinone	nitrogen-containing compound	872-50-4
oleamide	nitrogen-containing compound	301-02-0
benzothiazole*	sulfur-containing compound	95-16-9
dimethylsulfide*	sulfur-containing compound	3658-80-8

Schuh et al., Figure S1




Schuh et al., Figure S2
S2
T1A
T1B
T2
320
160
-160
-320
-320
-160
160
320
Component 2 (11.5 %)
Component 1 (53.7 %)

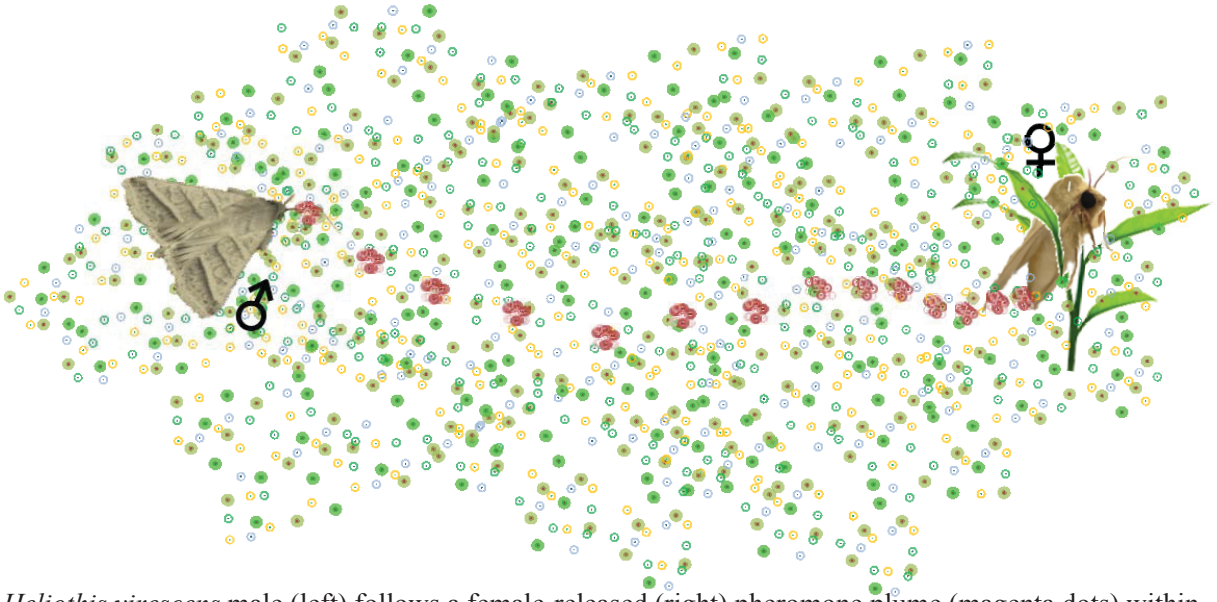
CHAPTER 3

A challenge for a male noctuid moth? Discerning the female sex pheromone against the background of plant volatiles

Elisa Badeke (Schuh), Alexander Haverkamp, Bill S. Hansson and Silke Sachse

'What we see is unforgetable. With a soft flick-flack the great Moths fly around the bell-jar, alight, set off again, come back, fly up to the ceiling and down. […] a memorable evening, this Great Peacock evening. Coming from every direction and apprised I know not how, here are forty lovers eager to pay their respects to the marriageable bride born that morning amid the mysteries of my study.'

Jean-Henry Fabre, 1879, translation of 'Souvenirs entomologiques' by A. T. de Mattos: The life of a caterpillar, chapter 11, 1916



Heliothis virescens male (left) follows a female-released (right) pheromone plume (magenta dots) within a background of plant volatile (green, yellow and blue dots).

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A Challenge for a Male Noctuid Moth? Discerning the Female Sex Pheromone against the Background of Plant Volatiles

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Finding a partner is an essential task for members of all species. Like many insects, females of the noctuid moth *Heliothis virescens* release chemical cues consisting of a species-specific pheromone blend to attract conspecific males. While tracking these blends, male moths are also continuously confronted with a wide range of other odor molecules, many of which are plant volatiles. Therefore, we analyzed how background plant odors influence the degree of male moth attraction to pheromones. In order to mimic a natural situation, we tracked pheromone-guided behavior when males were presented with the headspaces of each of two host plants in addition to the female pheromone blend. Since volatile emissions are also dependent on the physiological state of the plant, we compared pheromone attraction in the background of both damaged and intact plants. Surprisingly, our results show that a natural odor bouquet does not influence flight behavior at all, although previous studies had shown a suppressive effect at the sensory level. We also chose different concentrations of single plant-emitted volatiles, which have previously been shown to be neurophysiologically relevant, and compared their influence on pheromone attraction. We observed that pheromone attraction in male moths was significantly impaired in a concentration-dependent manner when single plant volatiles were added. Finally, we quantified the amounts of volatile emission in our experiments using gas chromatography. Notably, when the natural emissions of host plants were compared with those of the tested single plant compounds, we found that host plants do not release volatiles at concentrations that impact pheromone-guided flight behavior of the moth. Hence, our results lead to the conclusion that pheromone-plant interactions in *Heliothis virescens* might be an effect of stimulation with supra-natural plant odor concentrations, whereas under more natural conditions the olfactory system of the male moth appears to be well adapted to follow the female pheromone plume without interference from plant-emitted odors.

Keywords: *Heliothis virescens*, pheromone-guided flight behavior, plant volatiles, wind tunnel, GC-MS

INTRODUCTION

Odors present in the environment provide information that is crucial for insect survival and reproduction. Most insects use these olfactory cues for finding food, identifying suitable oviposition sites and communicating with their mates. Volatiles that are emitted by plants represent major cues with which an insect detects suitable host plants (Visser, 1986; Bruce et al., 2005), while pheromones are used for intraspecific identification and communication. Lepidoptera males, for example, are able to detect conspecific females releasing a species-specific pheromone blend. In the heliothine moth *Heliothis virescens* (Lepidoptera, Noctuidae), it has been shown that females produce a complex blend of up to seven components in their pheromone glands (Roelofs et al., 1974; Tumlinson et al., 1975; Klun et al., 1979; Pope et al., 1982). Wind tunnel and field experiments have shown that the behavioral activity of this pheromone blend depends highly on the ratio of its individual components (Vetter and Baker, 1983; Ramaswamy and Roush, 1986; Vickers et al., 1991). The pheromone blend is detected by specialized olfactory sensory neurons (OSNs) housed in sensilla trichoidea on the male antenna (Almaas and Mustaparta, 1990, 1991; Berg et al., 1995; Vickers et al., 2001). These OSNs send their axons to the antennal lobe (AL), which represents the primary olfactory processing neuropil, consisting of an array of olfactory glomeruli. Sex pheromone information is processed in a male-specific part of the AL (Hansson and Anton, 2000), the macroglomerular complex (MGC), which in male *Heliothis virescens* comprises four glomeruli (Christensen and Hildebrand, 1987; Hansson et al., 1992, 1995; Vickers and Baker, 1996; Berg et al., 1998; Vickers et al., 1998). The remaining, so-called ordinary, glomeruli process the information of all other odorants including plant and fruit volatiles (Galizia et al., 2000; Hillier and Vickers, 2007). This segregation of the olfactory pathway is partially maintained in the higher brain centers, such as the lateral horn (Zhao et al., 2014).

Heliothis virescens is a pest species, and feeds on many plants and crops such as cotton, tomato, soybean, tobacco and chickpea (Fitt, 1989; Cunningham and Zalucki, 2014). Several studies have shown that the olfactory system of both males and females is able to detect and process many volatiles emitted by these host plants (Loughrin et al., 1990; Tingle and Mitchell, 1992; Stranden et al., 2003; Rostelien et al., 2005; Hillier et al., 2006; Hillier and Vickers, 2007). Notably, the chemical diversity of volatile compounds found in all the floral scents investigated so far has been estimated to more than 1700 chemicals (Knudsen et al., 2006). Furthermore, the volatile composition of plants can change depending on environment and stress (reviewed by Dicke and Van Loon, 2000; Beyaert and Hilker, 2014). Damaged plants often emit different volatiles as well as different ratios of the volatile composition compared to undamaged plants. Considering this enormous diversity of chemical compounds, finding a sexual partner in such a complex environment is a big challenge for male moths. They have to detect minute amounts of the conspecific female pheromone blend against a constant background of many other odors. Although pheromone compounds are processed in a separate part of the olfactory system, it has been shown in several moth species that plant volatiles can influence pheromone detection and *vice versa* (Chaffiol et al., 2014; Deisig et al., 2014). Interestingly, plant compounds can even enhance the detection of pheromone components. For example, in the corn earworm *Helicoverpa zea*, simultaneous application of plant odorants with the major sex pheromone component of the moth increases the firing rate of pheromone-responsive OSNs in males, although those neurons do not respond to stimulation with plant odorants separately (Ochieng et al., 2002). Moreover, in beetles (Nakamuta et al., 1997) and many lepidopteran species (Dickens et al., 1993; Light et al., 1993; Reddy and Guerrero, 2000; Deng et al., 2004; Namiki et al., 2008; Schmidt-Büsser et al., 2009; Gurba and Guerin, 2015) the behavioral response is also increased when plant compounds are combined with the corresponding pheromone components. In contrast, a variety of studies demonstrated that pheromone detection can also be inhibited by interactions with plant odorants (Den Otter et al., 1978; Kaissling and Bestmann, 1989; Pophof and Van Der Goes Van Naters, 2002; Party et al., 2009, 2013; Hillier and Vickers, 2011; Chaffiol et al., 2012; Deisig et al., 2012; Pregitzer et al., 2012; Hatano et al., 2015). Hatano et al. (2015) showed this inhibitory effect even at the behavioral level. These contradictory findings give raise to the question whether the olfactory background is modulating the intraspecific communication of insects. Indeed, in *Heliothis virescens,* certain plant-emitted volatiles reduce the detection of *Z*11-16:Ald, the major sex pheromone component, at the level of the pheromone receptor HR13 (Pregitzer et al., 2012). Single sensillum recordings of *Z*11-16:Ald-tuned OSNs concur with this inhibitory effect (Hillier and Vickers, 2011). Moreover, in the same study, a suppressive effect for OSNs being tuned to the minor component *Z*9-14:Ald could be demonstrated. However, whether these effects at the sensory level are maintained throughout the olfactory system and thus may affect male moth behavior is unknown. We therefore analyzed whether a background of plant volatiles influences pheromone-guided behavior in *Heliothis virescens* using wind tunnel experiments. We analyzed the impact of complete and naturally occurring odor blends as well as of individual plant volatiles at different concentrations. Furthermore, we quantified the volatile emissions of all stimuli using gas chromatography analysis. Surprisingly, we observed pheromone-plant interactions only at high and supra-natural odor concentrations. We therefore conclude that pheromone-plant interactions in *Heliothis virescens* might not occur under natural conditions and that male moths are able to detect their conspecific female against a complex background of plant volatiles.

MATERIALS AND METHODS

Insect Rearing

We obtained *Heliothis virescens* from the Department of Entomology in the Max Planck Institute of Chemical Ecology in Jena. Moths originated from Clemson University in Clemson, South Carolina. These were maintained at the institute for several generations, where they were reared as follows: Eggs of *H. virescens* were gained from single pair matings in 0.5 l cups. In order to minimize inbreeding depression, females and

males of different families were chosen. A mesh on top of the mating cups allowed the females to oviposit their eggs. Larvae were subsequently maintained in 10-cm Petri dishes containing artificial pinto bean diet (Burton, 1979). They were separated at second instar. After eclosion, about 15–20 males of the same age were segregated into 30 × 30 × 30 cm rearing cages. A 10% sucrose solution was provided *ad libitum*. Animals were kept at 60% rel. humidity and at 23–25°C under a 16:8 h light-dark cycle. The light level during scotophase was 0.4 lux. 2- to 6-day-old virgin male moths were used for behavioral experiments.

Plant Material

In order to use the headspaces of whole plants for volatile collection and behavioral experiments, cotton (*Gossypium hirsutum*) and tomato plants (*Solanum lycopersicum*) were grown individually in 1-liter pots in the greenhouse at 23–25°C and 50–70% rel. humidity under a 16:8 h light-dark cycle. After the beginning of their elongation stage and until the experiments were performed, plants were transferred to a climate chamber providing 22–25°C and 60–70% relative humidity. They were watered daily with 100 ml tap water supplemented with 0.12 g*ml-1 fertilizer. For the experimental approach undamaged or damaged plants were taken. In order to damage plants, four to five third- and fourth-instar larvae of *H. virescens* were allowed to feed on the plant before the behavioral assay was conducted. Larvae were removed from the plants after 24 h.

Behavioral Approach

Wind Tunnel

Insects were tested in a 220 × 90 × 90 cm Plexiglas wind tunnel (**Figure 1A**) under infrared and red light conditions with a white light supply of 0.4 lux. A purified, humidified and tempered airflow of 0.27 m/s was blown through the wind tunnel, providing 23°C and 60–70% relative humidity.

Stimulus Device

For synthetic odorants the odor plume was created by connecting separately two 50 ml glass bottles via *Teflon* tubing to the stimulus outlet on a stick 55 cm long (**Figure 1**). The distance to the upwind end of the wind tunnel was 23 cm. Pumps, which sucked the ambient air through a charcoal filter for cleaning, generated a stimulus flow of 0.48–0.50 l/min through the tubing leaving

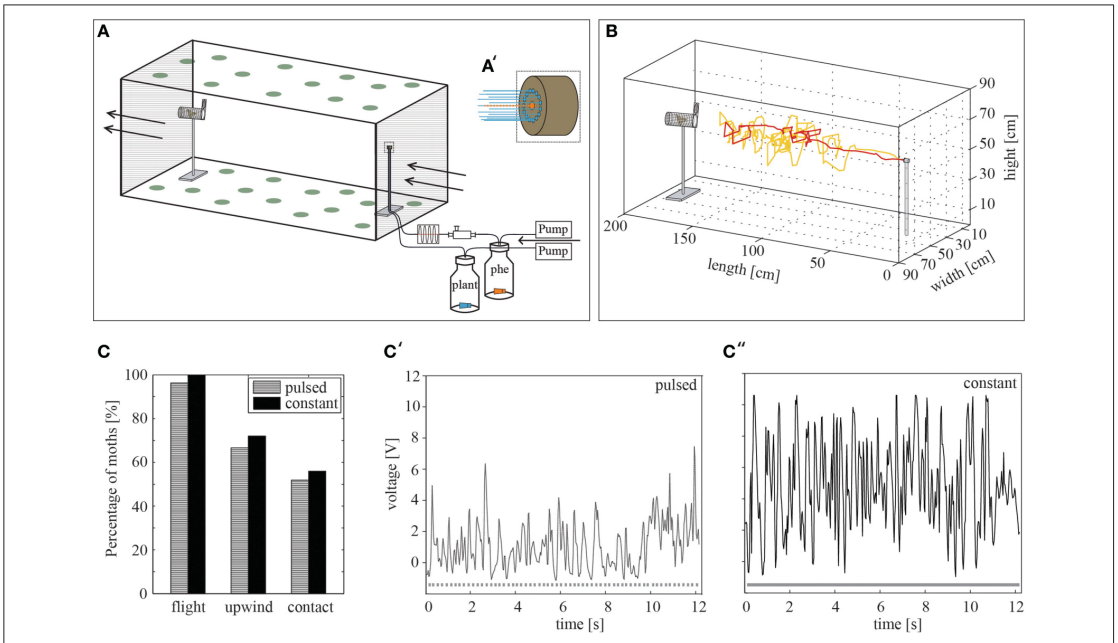

FIGURE 1 | The wind tunnel system. (A) Schematic representation of the wind tunnel system including the stimulus device. The ceiling and the floor were covered by green dots in order to provide a pattern for the insects to orient on. Arrows indicate the air stream. An air flow is transported via pumps through the stimulus bottles and released by the stimulus outlet. The pheromone-loaded air is pulsed beforehand at 10 Hz by using a cross-valve. phe = pheromone **(A')** Magnification of the stimulus outlet (dashed square). The dotted orange line represents the middle nozzle, which emits a pulsed pheromone stimulus, while the blue lines highlight the constant plant odor flow released by the surrounding nozzles. **(B)** Two representative flights of different males (yellow, red) toward the pheromone blend. **(C)** The percentage of male *H. virescens* attempting flight behavior, achieving upwind flight and making source contact is similar for constant ($N = 25$) and pulsed ($N = 27$) pheromone stimulation ($p > 0.05$, Fisher's exact test). **(C',C'')** Visualization of the constant and pulsed odor plume using a photoionization detector (PID) at 110 cm distance from the stimulus outlet. Dotted and continuous lines below the curves represent the odor stimulation. Fewer volatiles can be detected in the pulsed **(C')** odor plume than in the constant plume **(C'')**. PID measurements: $U_{pulsed} = 1.81$ V, $U_{constant} = 4.77$ V.

the bottles. In each of the bottles, a rubber septum loaded with the test odorants was inserted. The bottle, which contained the pheromone blend, was additionally connected to an *Arduino* microprocessor-controlled cross-valve before being released by the middle nozzle (ID 1 mm) of the stimulus outlet (**Figure 1A′**). Thus, pulsed stimulations of 10 Hz could be achieved. It has been shown that pulsed stimulation affects the flight behavior of male moths in the wind tunnel (Vickers and Baker, 1994). We therefore compared pheromone attraction to either a constant pheromone plume or a pulsed pheromone plume using an optimal pulse frequency of 10 Hz (**Figure 1C**). The second stimulus bottle was connected to the circular arranged nozzles (ID 0.5 mm each). For experiments using the headspaces of different plants, a glass cylinder (10 l) containing a plant was connected to the system instead of to the second stimulus bottle. A Teflon disc on the bottom with a central opening separated green plant material from soil and roots. Compressed, charcoal-filtered air with a flow of 1 l/min was inserted into the cylinder. Only 0.48–0.54 l/min of the cylinder headspace was sucked via a pump into to the wind tunnel.

Animal Handling

All experiments were performed 2–7 h during scotophase, when pheromone responsiveness is highest (Shorey and Gaston, 1965). At least 1 h before testing, male moths were transferred individually into Ø 7 × 10 cm mesh tubes and placed in a small room near the wind tunnel that had the same conditions. Active moths were chosen for testing. At the beginning of each experiment, a mesh tube containing a moth was inserted into a releasing device in the odor plume at the downwind end of the wind tunnel. The releasing device was controlled via the microprocessor in order to open the cage automatically 2 min after placing the moth in the mesh tube. Flight behavior was subsequently recorded for 5 min. After the first source contact within this time interval, males' behavior was tracked for 2 min.

3-D Video Tracking

During the experiment the releasing device, all wind tunnel conditions and the flight paths were computer-controlled from a separate room. In order to observe odor-guided flight behavior, we used a custom-built video tracking system. Four cameras (C615, Logitech, Newark, NJ, USA, 800 × 600 pixels, 0.3 cm^2 pixel size), which were located at the side and on the top of the wind tunnel, recorded the flight path of each moth. By using a background subtraction algorithm, the position of each moth was calculated at a rate of 10 Hz. A fifth camera, which was attached to the upwind end of the wind tunnel, allowed the recording of males' behavior close to the odor source.

Determining Optimal Conditions for the Wind Tunnel

In order to monitor pheromone attraction and to study whether it is influenced by background volatiles, we started to find the best conditions for the bioassay. A stimulus device was used to create a point source emitting either a pulsed or a constantly emitted pheromone blend of *Heliothis virescens* together with a surrounding odor plume of a constant solvent release (**Figure 1A′**). When stimulating with the conspecific pheromone blend, male moths showed clear pheromone-guided upwind flight behavior. This behavior can be characterized by locking on to the pheromone plume followed by upwind flight, zigzagging, casting behavior and, finally, contact with the source (**Figure 1B**). When placed in a constant or a pulsed pheromone plume, all moths started their flight within 5 min. (**Figure 1C**). Hence, the type of stimulation influenced neither the percentage of moths attempting upwind flight nor the number of source contacts. In order to compare the pulsed and constant odor plume structure, we measured the presence of volatiles using a photoionization detector (PID). The results showed that the probability that a moth hits a volatile in a pulsed odor plume is less than the probability that a moth hits one in a constant plume (**Figures 1C′,C″**). However, although the odor plume structure was different, pheromone attraction was similar for both odor applications. We chose pulsed pheromone stimulation for all subsequent experiments in our study.

Odorants

All synthetic odorants tested were commercially available and acquired from Sigma (http://www.sigma-aldrich.com), Bedoukian (http://www.bedoukian.com) or pherobank (http://www.pherobank.com). They were obtained in the highest available purity. β-caryophyllene (CAS 87-44-5, purity > 98.5%), racemic linalool (CAS 78-70-6, purity > 97%) and (*Z*)3-hexen-1-ol (CAS 928-96-1, purity > 98%) are well-described plant compounds. They are detectable by male and female *Heliothis virescens* (Paré, 1997; De Moraes et al., 2001; Skiri et al., 2004; Rostelien et al., 2005; Hillier and Vickers, 2007), and they have been used previously in studies investigating plant-pheromone interaction on *H. virescens* (Dickens et al., 1993; Hillier and Vickers, 2011; Pregitzer et al., 2012).

A synthetic pheromone blend, which contained the seven components, (*Z*)-11-hexadecenal (*Z*11-16:Ald, CAS 53939-28-9, purity 97-98%), (*Z*)-9-tetradecenal (*Z*9-14:Ald, CAS 53939-27-8, purity > 93%), tetradecenal (14:Ald, purity > 98%), hexadecanal (16:Ald, CAS 629-80-1, purity > 93%), (*Z*)-7-hexadecenal (*Z*7-16:Ald, CAS 56797-40-1, > 95%), (*Z*)-9-hexadecenal (*Z*9-16:Ald, CAS 56219-04-6, purity > 90%) and (*Z*)-11-hexadecenol (*Z*11-16:OH, CAS 56683-54-6, purity > 98%), was used (Roelofs et al., 1974; Tumlinson et al., 1975; Klun et al., 1979). We prepared the blend relative to *Z*11-16:Ald (100%) and added 5% *Z*9-14:Ald, 5% 14:Ald, 10% 16:Ald, 2% *Z*7-16:Ald, 2% *Z*9-16:Ald and 1% *Z*11-16:OH of the compounds (Pope et al., 1982), in order to test the sexual attraction of *H. virescens* males toward their conspecific pheromone blend. Tetradecenal was synthesized from commercially available tetradecanol (Sigma) by the Research Group Mass Spectrometry/Proteomics in the Max Planck Institute of Chemical Ecology in Jena.

Both synthetic plant compounds and the pheromone blend consisted additionally of 1.25% of the antioxidant 3.5-Di-tert-butyl-4-hydroxytoluene (BHT, CAS 128-37-0, purity ≥ 99%, Sigma). They were subsequently pipetted on individual rubber septa (Thomas Scientific, http://www.thomassci.com/). Before being used, rubber septa were cleaned with hexane (CAS 110-54-3, Sigma), which was furthermore used as a solvent for all odorants. For plant components, concentrations between 30 and

300 μg/μl were used. The pheromone blend was adjusted to *Z*11-16:Ald with a concentration of 300 μg/μl. We always indicate the final concentration for each rubber septum.

Volatile Collection, Analysis, and Quantification

In order to quantify the actual amount of volatiles being released by the rubber septum and pumped through the tubing into the wind tunnel, we used polydimethylsiloxane (PDMS) tubes (OD 2.3 mm, Reichelt Chemietechnik, http://www.rct-online.de). By introducing the PDMS tubes for 2 h into the odor flow close to the stimulus outlet, we could collect volatiles during testing. Volatiles being released by plants were collected with the same approach. Samples were stored at -20°C until use. All samples were examined on an Agilent 7890A gas chromatograph (Agilent Technologies, CA) running in splitless mode and being connected to an Agilent 5975C mass spectrometer (electron impact mode, 70 eV, ion source: 230°C, quadrupole: 150°C, mass scan range: 33–350 u). We used a nonpolar column (HP-5 MS UI, 30 m length, 0.25 mm ID, 0.25 μm film thickness, J and W Scientific) under constant helium flow of 1.1 ml/min. The GC oven was programmed to hold 40°C for 3 min, to increase the temperature at 5C°/min to 200°C, then to increase temperature at 20°C/min to 260°C. The maximum temperature was held for 10 min. For identification, mass spectra were compared with Kovats retention time indices to reference compounds or to those published by the National Institute of Standards and Technologies (NIST, version 2.0). Retention times for all compounds were determined by using standards. Quantifications of emission rates were subsequently calculated based on the comparison of the internal standard of 10 ng/μl 1-Bromohexane (CAS 111-25-1, purity 98 %, Sigma) and peak area of single compounds.

Data Analysis and Statistics

Microsoft Excel, Gnu R, custom-written Matlab scripts (MATLAB version- Mathworks, USA) and Adobe Illustrator were used in order to analyze and plot all data. Statistics were performed with the software Gnu R and GraphPad Instat. We calculated the emission rate of volatiles being released within 1 h for each compound based on the internal standard by using the commercial software GC ChemStation (Agilent Technologies) and Microsoft Excel.

In order to investigate the attractiveness of volatiles in the wind tunnel, we calculated the percentage of moths (1) starting to fly, (2) achieving upwind flight, and (3) contacting the source for each group of odor stimulation. An odor plume was called attractive if moths reached and contacted the odor source. In order to investigate pheromone-plant interaction, we further examined the average number of source contacts per male out of all individual moths within a group for the test period. We quantified the number of contacts for another 2 min after the first contact. Males without contacts were counted as zeros. For statistical analysis, the group tested with the pheromone blend alone was always taken as a control group. The percentage of moth within a test group was compared to the pheromone group by means of Fisher's exact test, with a Bonferroni-Holm correction. The number of source contacts was evaluated using the Kruskal-Wallis test with Dunn's multiple comparisons test. The pheromone-guided flight behavior of each attracted male was analyzed in more detail by calculating the percentage of relative abundance of flight angles in y- and z-direction and the average upwind speed within an 80 cm distance from the stimulus outlet. Both angles and upwind speed were measured with an interval of 10 Hz. The last 10 cm of the track were excluded due to the fact that it could not be tracked reliably in all moths. Animals which performed zigzagging and casting movements possessed flight angles greater than zero degrees. Angles around zero degrees exhibit straight upwind movement. Upwind speed (cm/s) is the speed of an animal relative to the odor source. Positive values indicate upwind movement, negative values downwind movement, while values around zero indicate cross-wind movement. The Kruskal-Wallis test and Dunn's multiple comparisons test were used for statistics.

RESULTS

Host Plant Headspaces Did Not Affect Pheromone Attraction

Since it has been shown that different plant-emitted volatiles affect detection of the major sex pheromone component *Z*11-16:Ald in male *Heliothis virescens* at the physiological level (Hillier and Vickers, 2011; Pregitzer et al., 2012), we tested whether behavioral performance is similarly affected. In order to provide a naturally occurring odor source, we used the headspaces of two host plants, tomato and cotton, to examine their influence on pheromone-guided flight behavior (**Figure 2A**, left panel). First, we tested the headspaces of the two host plants alone. We observed that both the tomato headspace as well as the cotton headspace induced only very low degrees of upwind flight and source contact ($N = 17$–20, upwind 1–3 moths, contact 0–1 moth; data not shown). We next applied the conspecific pheromone blend to each plant headspace simultaneously. The results reveal that a pheromone plume with a background of either tomato (**Figure 2A**, middle panel) or cotton headspace (**Figure 2A**, right panel) showed similar attractiveness as compared to a pheromone blend with no plant odor background. The number of source contacts was also not affected (**Figure 2C**, **Table 1**). Hence the pheromone-guided flight was not influenced by the presence of a naturally occurring plant odor blend.

It has been shown that larval damage influences the composition and/or the emission rate of plant volatiles (De Moraes et al., 1998). The attraction of female moths to a damaged plant headspace depends on the amount of herbivore-induced plant volatiles (Späthe et al., 2013). In order to examine whether herbivore damage significantly influences pheromone detection, we let four to five larvae feed on both host plants and tested the attractiveness of the induced headspace in our wind tunnel. Only three moths at most moved upwind when placed in a damaged tomato or cotton odor plume, but none of them contacted the source ($N = 15$–17; data not shown). When a damaged tomato plant headspace was

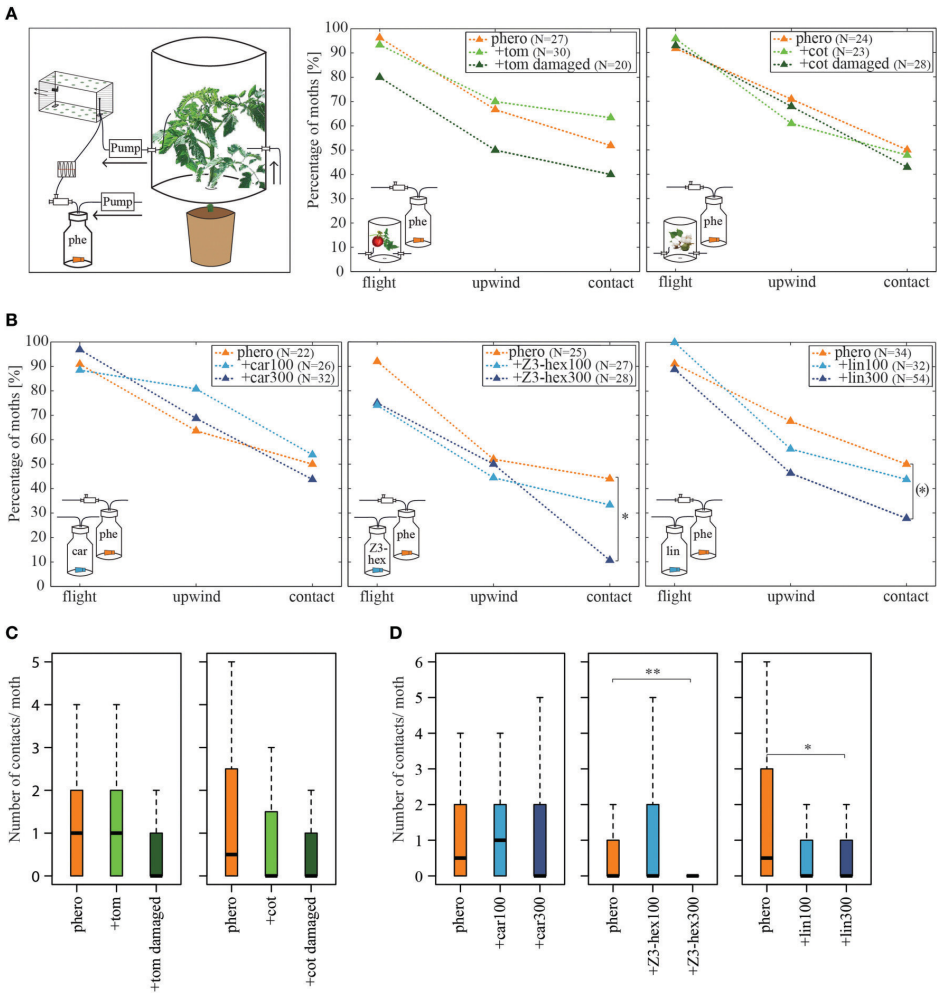

FIGURE 2 | Influence of host plant headspaces on pheromone-guided flight behavior. (A) Percentage of moths attempting flight behavior, achieving upwind flight and making source contact, when simultaneously stimulated with the pheromone blend and a tomato (middle panel) or cotton (right panel) plant headspace. Plants were intact or damaged by larvae. The left panel highlights the changes in the odor stimulation device. The headspace of the plants was sucked via a pump through the wind tunnel. The pulsed pheromone stimulation was implemented as described in **Figure 1**. There was no significant difference in pheromone attraction when insects were stimulated simultaneously with undamaged or damaged tomato or cotton headspaces compared to pheromone stimulation alone ($p > 0.05$, Fisher's exact test, Bonferroni-Holm correction). **(B)** Percentage of moths attempting flight behavior, achieving upwind flight and making source contact, when simultaneously stimulated with the pheromone blend and the synthetic odorants β-caryophyllene (left panel), (*Z*)3-hexenol (middle panel) or linalool (right panel) each in two different concentrations (100 and 300 μg/μl). While β-caryophyllene did not affect pheromone-guided flight behavior, high concentrations of (*Z*)3-hexenol decreased the amount of moths contacting the source. A similar tendency was observed for linalool. Asterisks represent significant differences ($p < 0.05$, Fisher's exact test with Bonferroni-Holm correction). The bracket indicates significant differences without Bonferroni-Holm correction ($p = 0.0426$). **(C)** Number of contacts per individual moth for all tested males from **(A)**. No differences in the number of contacts when different plant headspaces were used ($p > 0.05$, the Kruskal-Wallis test, Dunn's multiple comparisons test). **(D)** Number of contacts per individual moth for all tested males from **(B)**. Moths had significantly fewer contacts when high dosages of (*Z*)3-hexenol or linalool were applied to the septa than when they were not ($p < 0.05$, the Kruskal-Wallis test, Dunn's multiple comparisons test). car, β-caryophyllene; cot, cotton; lin, linalool; phe/phero, pheromone; tom, tomato; *Z*3-hex, (*Z*)3-hexenol.

TABLE 1 | Effect of intact and damaged tomato and cotton plants on pheromone-guided flight behavior.

Stim. 1	Stim. 2	Sample size	Flight [%]	Upwind [%]	Source contact [%]	Upwind speed [cm/s] ± SD	Number of contacts ± SD
–	Phero	27	96.3	66.7	51.9	25.8 ± 29.6	1.19 ± 1.71
Tom	Phero	30	93.3	70	63.3	22.7 ± 27.3	1.37 ± 1.56
Tom damaged	Phero	20	80	50	40	24 ± 22.7	0.6 ± 0.99
–	Phero	24	91.7	70.8	50	24 ± 22.7	1.75 ± 2.67
Cot	Phero	23	95.7	60.9	47.8	30.5 ± 23	1 ± 1.38
Cot damaged	Phero	28	92.9	67.9	42.9	25.1 ± 33.6	0.75 ± 1.17

*Number of tested individuals and the percentages of male moths, for the experiments shown in **Figures 2A,C**, which started their flight, showed upwind movement and had source contact; also their upwind speed. The last column represents the number of contacts for all tested males. Stimulus (stim.) 1 and 2 together form the odor plume. Odorants of stimulus 1 were emitted continuously, while stimulus 2 (pheromone) was pulsed. A (–) in stimulus 1 represents the use of a solvent instead of an odorant. SD, standard deviation.*
no significant differences within a column to the solvent-pheromone stimulation ($p > 0.05$, Fisher's exact test with Bonferroni-Holm correction; Number of contacts and upwind speed: Kruskal-Wallis with Dunn's multiple comparisons test).
cot, cotton; phero, pheromone; tom, tomato.

presented together with the pheromone blend, we observed that 12% fewer individuals reached the source as compared to the pure pheromone blend (**Figure 2A**, middle panel, **Table 1**). However, this decrease was not significantly different from the response to the pheromone blend without background. Likewise, moths flying in a pheromone plume did not contact the source significantly more often (**Figure 2C**, **Table 1**). The same applies for the cotton headspace: larval damage in cotton plants affected neither pheromone-guided flight behavior nor the number of odor source contacts (**Figure 2A**, right panel, **Figure 2C**, **Table 1**).

In order to analyze pheromone-guided flight behavior in more detail, we dissected the flight mechanism. We asked how males manoeuver in response to an odor source and if their flight patterns are influenced by different odor plumes. We therefore examined the flight angles of attracted individuals as well as individual's upwind speed (**Figure 3**). In **Figure 3A** the relative abundance of flight angles for male moths in a pure pheromone plume and a tomato-pheromone plume are representative examples. Independent of odor stimulation, the most abundant flight angles of male *Heliothis virescens* were around zero degrees, indicating a relatively straight upwind flight. Angles up to $\pm 180^{\circ}$ represented additional zigzagging and casting behavior. Analysis of the upwind speed of the attracted insects resulted in values around 27 cm/s regardless of the odors present in the plume (**Figure 3B**, **Table 1**). In summary, we observed that neither the number of source contacts nor the flight pattern was affected when a complete plant headspace was applied simultaneously with the pheromone blend.

Certain Plant-Emitted Volatiles Reduced Pheromone Attraction

Interestingly, we did not observe the significant reduction in pheromone-elicited flight behavior suggested in previous studies. These however reported plant-pheromone interactions in moths using single plant-related compounds instead of complete headspaces. In order to analyze whether single plant volatiles could affect the pheromone response, we tested the three plant-emitted volatiles, β-caryophyllene, (*Z*)3-hexenol and linalool, each in two different concentrations based on the study by Pregitzer et al. (2012). As a side note, all of these compounds are up-regulated in larval-damaged plants (Paré, 1997; De Moraes et al., 1998, 2001; Stranden et al., 2003; Morawo and Fadamiro, 2014).

In comparison to pure pheromone stimulation, both concentrations of β-caryophyllene in combination with the pheromone did not reduce the attractiveness of the pheromone (**Figures 2B,D**, left panels, **Table 2**); moreover, β-caryophyllene alone did not attract any male moths, independent of its concentration (tested concentrations: 60, 100, 200, 300 µg/µl; $N = 16$–19; data not shown). Likewise, male moths did not respond to (*Z*)3-hexenol alone (100, 300 µg/µl; $N = 16$; data not shown). However, adding 300 µg/µl of (*Z*)3-hexenol to the pheromone plume significantly reduced the number of individuals (by 33%) and their frequency contacting the source, although equal percentages displayed upwind flight (**Figures 2B,D,** middle panels, **Table 2**). Interestingly, lowering the concentration of (*Z*)3-hexenol (i.e., 100 µg/µl) did not significantly decrease the moths' response to pheromones. We observed a similar dose-dependent effect when insects were stimulated simultaneously with the pheromone blend and the odor linalool. Linalool alone at concentrations of 30, 60, 100, 200, or 300 µg did not attract males at all and resulted in no upwind flights ($N = 15$–30; data not shown). However, adding the highest concentration of linalool to the pheromone plume resulted in 22% fewer individuals contacting the source compared to the number contacting the source when only the pheromone was used (**Figures 2B,D**, right panels, **Table 2**). This effect was also concentration-dependent, since we did not observe any reduction in pheromone-guided flight behavior when we reduced the concentration of linalool.

We observed similar flight angles in a pheromone plume compared to those in a plume consisting of the pheromone blend and β-caryophyllene, (*Z*)3-hexenol or linalool, as shown for β-caryophyllene and (*Z*)3-hexenol (**Figure 3A**, **Table 2**). The distribution histograms represent the cumulated azimuth and zenith angles of all male moths contacting the stimulus outlet. Since we measured less animals for (*Z*)3-hexenol, the histogram shows less cumulated angles. However, the distribution of the angles is similar to those of the other stimuli. Most angles were around zero degrees. Furthermore, males moved upwind to the

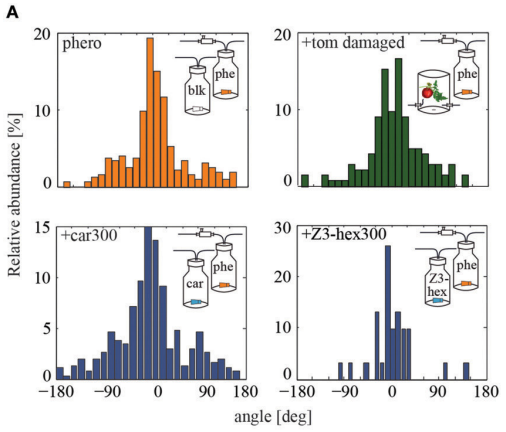

FIGURE 3 | Influence of individual plant volatiles on pheromone-guided flight behavior. (A) Relative abundance of flight angles being performed by male moths within the upwind section of the wind tunnel close to the source, when pheromones alone (orange), a pheromone and the headspace of a damaged tomato plant (dark green), pheromone and β-caryophyllene or (*Z*)3-hexenol (dark blue, both with 300 µg), were used for stimulation. The distribution histograms cumulate all tracks of moths contacting the stimulus outlet. Flight angles are shown as cumulated azimuth and zenith angles. Most of the time insects facing upwind in the odor plume they perceived performed zigzagging and casting activities. **(B)** Males moths showed similar upwind speeds ($p > 0.05$, the Kruskal-Wallis test, Dunn's multiple comparisons test), when stimulated with the pheromone alone (orange, $N = 41$), or the pheromone in combination with 100 µg ($N_{car} = 12$, $N_{Z3-hex} = 8$, $N_{lin} = 9$) or 300 µg ($N_{car} = 13$, $N_{Z3-hex} = 3$, $N_{lin} = 14$) of artificial odorants (blue), or with the headspaces of cotton ($N_{intact} = 8$, $N_{dam} = 13$), or tomato plants ($N_{intact} = 10$, $N_{dam} = 7$) (green). blk, blank; car, β-caryophyllene; cot, cotton; lin, linalool; phe/phero, pheromone; tom, tomato; *Z*3-hex, (*Z*)3-hexenol.

source with on average 25 cm/s (**Figure 3B**). In summary, adding certain plant-related compounds at high concentration to the pheromone plume reduced the pheromone-guided response in male *Heliothis virescens* but did not lead to a different flight pattern: neither the flight direction in order to approach the odor source nor the upwind speed was influenced by plant volatiles.

Concentration Quantification of Synthetic Odorants vs. Plant-Released Volatiles

Our experiments show that only the application of linalool and (*Z*)3-hexenol at high concentration reduced the attractiveness of male *Heliothis virescens* to the pheromone, while the headspace of host plants did not show any influence. In order to analyze whether the difference is just a matter of odor concentration, we quantified the actual amount of the synthetic odorants released by the rubber septa (**Figures 4A,B**). While 3 ng of the major sex pheromone component *Z*11-16:Ald could be quantified via PDMS tubes, the plant components, β-caryophyllene, (*Z*)3-hexenol and linalool, were measured in much higher amounts. The amount of β-caryophyllene was 3.5-fold higher than the amount of (*Z*)3-hexenol, while the linalool release was 5-fold higher than the amount of (*Z*)3-hexenol. When pipetting three times the concentration on a rubber septum, both plant volatiles resulted in doubled emission rates, while only 1.5-fold of linalool was detected.

Are the synthetic single odor quantities that reduced the attractiveness of pheromones in our wind tunnel studies similar to those released by intact and damaged tomato and cotton plants? To find out, we quantified the release rate of β-caryophyllene, (*Z*)3-hexenol and linalool in damaged and undamaged host plants (**Figure 4C**). Larval damage in tomato and cotton plants led to an increase of β-caryophyllene (**Figure 4C**, left panel), and β-caryophyllene was released in quantities comparable to those of the synthetic odorant. However, β-caryophyllene had no effect on pheromone-guided flight behavior in male moths (**Figure 2A**). In contrast, (*Z*)3-hexenol and linalool could not be detected in undamaged plants or were found in only low quantities in damaged plants (**Figure 4C**, middle and right panels). This discrepancy shows that the concentrations of (*Z*)3-hexenol and linalool that reduced pheromone attraction (**Figure 2A**) were much higher than the natural emission of an entire plant. Hence, odorants that influence pheromone-guided behavior in male moths are not emitted in comparable quantities by plants. We therefore conclude that plant-pheromone interactions in *Heliothis virescens* most likely occur only under laboratory conditions, where very high odor concentrations are used.

DISCUSSION

We show that pheromone-plant odor interactions occur at the behavioral level of male *Heliothis virescens*, similar to those previously observed at the sensory level (Hillier and Vickers, 2011; Pregitzer et al., 2012). However, we also show that these interactions occur only at supra-natural concentrations of certain plant-emitted volatiles. Our findings therefore suggest that, in a natural environment, male moths are able to detect their conspecific female against a complex background of plant volatiles without negative effects on their pheromone-directed flight behavior.

Certain plant-related volatiles interfere with the detection of the major sex pheromone component of *Heliothis virescens* at the pheromone receptor HR13 and thereby reduce the

TABLE 2 | Effect of β-caryophyllene, (*Z*)3-hexenol and linalool on pheromone-guided flight behavior.

Stim. 1	Stim. 2	Sample size	Flight [%]	Upwind [%]	Source contact [%]	Upwind speed [cm/s] ± SD	Number of contacts ± SD
–	Phero	22	90.1	63.6	50	22.6 ± 25.5	1 ± 1.23
car100	Phero	26	88.5	80.8	53.8	28.9 ± 28	1.04 ± 1.22
car300	Phero	32	96.9	68.8	43.8	24.7 ± 33.4	1.09 ± 1.53
–	Phero	25	92	52	44	29.1 ± 21.8	1.16 ± 1.84
Z3-hex100	Phero	27	74.1	44.4	33.3	31.1 ± 28.2	1 ± 1.71
Z3-hex300	Phero	28	75	50	10.7*	30.2 ± 28	0.27 ± 0.93**
–	Phero	34	91.2	67.6	50	15.8 ± 30.2	1.32 ± 1.66
lin100	Phero	32	100	56.3	43.8	16.1 ± 40.4	0.94 ± 1.9
lin300	Phero	54	88.9	46.3	27.8 (*)	23.7 ± 34.5	0.63 ± 1.51*

*Number of tested individuals and the percentages of male moths, for the experiments shown in **Figures 2B,D**, which started their flight, showed upwind movement, and had source contact; also, their upwind speed. The last column includes the number of contacts for all tested males. The stimuli were applied as described in **Table 1**. SD, standard deviation.*
**Within a column indicate significant differences to the solvent-pheromone stimulation (*$p < 0.05$, **$p < 0.01$, Fisher's exact test with Bonferroni-Holm correction, (*) $p < 0.05$, Fisher's exact test, $p > 0.025$ with Bonferroni-Holm correction; Number of contacts and upwind speed: Kruskal-Wallis with Dunn's multiple comparisons test).*
car, β-caryophyllene; lin, linalool; phero, pheromone; Z3-hex, (Z)3-hexenol.

response of pheromone-detecting OSNs in the MGC (Pregitzer et al., 2012). Interestingly, this interference varies for different plant compounds: linalool and (Z)3-hexanol strongly suppress the pheromone response, while other compounds, such as β-caryophyllene, do not lead to any reduction. These findings correlate well with our behavioral results from experiments using the wind tunnel: while β-caryophyllene did not influence pheromone-guided flight behavior, high concentrations of (*Z*)3-hexenol and linalool reduced the attractiveness of the pheromone by at least 22%. Hence our results show that the coding of pheromone-plant interactions at the sensory level corresponds to the altered behavioral responsiveness of male moths. The representation of odor-induced activity in the AL therefore allows a prediction of the behavioral outcome. Notably, a correlation between the representation of odors in the AL and the behavioral performance has already been demonstrated in several species, such as honeybees (Guerrieri et al., 2005), flies (Knaden et al., 2012) and moths (Kuebler et al., 2012).

The behavioral performance of the moth ultimately results from the odor representation in higher brain centers and is determined by the integration of different processing channels within the neuronal network. Interestingly, when the antenna of the male *Heliothis virescens* moth was stimulated with β-caryophyllene and the major sex pheromone component *Z*11-16:Ald, single sensillum recordings showed an enhanced spiking activity compared to the response evoked by *Z*11-16:Ald alone (Hillier and Vickers, 2011). In contrast, when the major pheromone component was exchanged for the minor pheromone component, *Z*9-14:Ald, the pheromone response was suppressed (Hillier and Vickers, 2011). Although β-caryophyllene is influencing the neuronal activity of pheromone-responsive OSNs in the periphery, we did not observe any effect of this plant volatile onto the pheromone-guided flight behavior in our windtunnel experiments. Since β-caryophyllene modulates the major and minor pheromone pathways in opposing directions (Hillier and Vickers, 2011), the detection of the whole pheromone blend, including the two compounds, *Z*11-16:Ald and *Z*9-14:Ald, might not be modulated in the end.

Moreover, in the same physiological study (Hillier and Vickers, 2011), both major and minor sex pheromone components, when blended with the plant volatile linalool or (*Z*)3-hexenol, elicited reduced spiking activity in the corresponding pheromone-responsive OSNs. Likewise, in our wind tunnel assay, when high concentrations of the two plant compounds were added, the attractiveness of the complete pheromone blend was decreased, which resulted in reduced pheromone-guided flight behavior.

The three compounds that we used in our study are not the only volatiles being detected in plant headspaces. It would therefore be interesting to know if and how other plant volatiles, when added to the pheromone blend, influence the pheromone-guided behavior of a moth. This is of particular interest, since it has been observed that some of these green leaf volatiles increase the number of males caught in pheromone traps (Dickens et al., 1993). However, when we tested the whole headspaces of cotton and tomato plants, independently of their physiological condition, we did not find any influence on pheromone-guided flight behavior.

Host plants of *Heliothis virescens* that are damaged by larval feeding release volatiles such as β-caryophyllene, (*Z*)3-hexenol and linalool (e.g., Paré, 1997; De Moraes et al., 1998; Morawo and Fadamiro, 2014). All of these were used in our study. When we quantified the natural emission of these compounds, we realized that, except for β-caryophyllene, these odorants occur in only very low concentrations in the headspace of intact or damaged cotton and tomato plants. Although volatiles are usually emitted in higher amounts during daytime than in the dark (De Moraes et al., 2001), male moths are active in the scotophase. Therefore, they will encounter low concentrations of plant volatiles. When the results from the wind tunnel and GC-MS experiments were combined, we observed that unnaturally high concentrations of (*Z*)3-hexenol and linalool reduced the heliothine moths' attraction to pheromones, while a lower dose, which represents the more natural situation, did not affect the attraction.

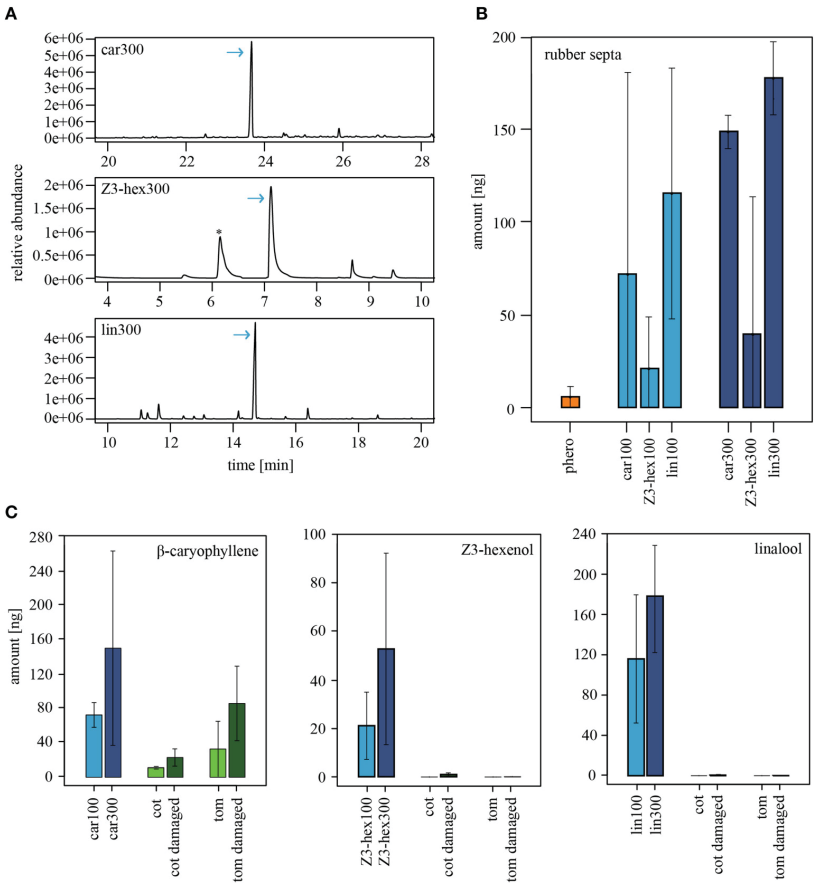

FIGURE 4 | Emission rates of volatiles from rubber septa and entire plants. (A) GC-MS example traces showing the relative abundance of the synthetic odorants β-caryophyllene (upper panel), (*Z*)3-hexenol (middle panel) or linalool (lower panel). 300 μg/μl of the odorant were loaded on a rubber septum, and the headspace was collected for 2 h with PDMS tubes. Arrows indicate the corresponding odor peak in the headspace. The asterisk represents the peak of a siloxane, which is a constituents of PDMS tubes. **(B)** Amounts of volatiles, which were released by rubber septa loaded with the pheromone blend ($N_{300\mu g} = 2$), β-caryophyllene ($N_{100\mu g} = 3$, $N_{300\mu g} = 3$), (*Z*)3-hexenol ($N_{100\mu g} = 2$, $N_{300\mu g} = 2$), or linalool ($N_{100\mu g} = 3$, $N_{300\mu g} = 3$). The averaged emission rates are shown as bar plots (±SEM). Bars represent odorants used in a concentration of 100 μg/μl (light blue) or 300 μg/μl (dark blue). **(C)** Comparison of the odor amount emitted from the rubber septa shown in **(B)** and the corresponding compounds in the plant headspace of intact (light green) and damaged (dark green) tomato ($N_{intact} = 2$, $N_{dam} = 4$) and cotton plants ($N_{intact} = 2$, $N_{dam} = 4$). Bars represent the averaged emission rates. Similar amounts of β-caryophyllene (left panel) were found in the odor emitted from the rubber septa and in odors released by the plants. (*Z*)3-hexenol (middle panel) and linalool (right panel) released from the plants were either not detected or occurred in low amounts that were not comparable to the amounts being released by the rubber septa. car, β-caryophyllene; cot, cotton; lin, linalool; phero, pheromone; tom, tomato; *Z*3-hex, (*Z*)3-hexenol.

Taken together, our study underlines the importance of using natural concentrations in order to investigate the ecological relevance of odorants and their influence on animals' behavior.

AUTHOR CONTRIBUTIONS

EB and SS together conceived and designed the study. EB planned and carried out all experiments. EB and SS analyzed and interpreted the results, prepared the figures and wrote the paper. AH helped to analyze the windtunnel data. BSH provided intellectual and financial support. All authors critically revised the article.

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CHAPTER 4

Electrical synapses mediate synergism between pheromone and food odors in Drosophila melanogaster

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Male and female of *Drosophila melanogaster* mate on an apple. Photo: B. Fabian

Electrical synapses mediate synergism between pheromone and food odors in *Drosophila melanogaster*

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In *Drosophila melanogaster*, the sex pheromone produced by males, *cis*-vaccenyl acetate (cVA), evokes a stereotypic gender-specific behavior in both males and females. As *Drosophila* adults feed, mate, and oviposit on food, they perceive the pheromone as a blend against a background of food odors. Previous studies have reported that food odors enhance flies' behavioral response to cVA, specifically in virgin females. However, how and where the different olfactory inputs interact has so far remained unknown. In this study, we elucidated the neuronal mechanism underlying the response at an anatomical, functional, and behavioral level. Our data show that in virgin females cVA and the complex food odor vinegar evoke a synergistic response in the cVA-responsive glomerulus DA1. This synergism, however, does not appear at the input level of the glomerulus, but is restricted to the projection neuron level only. Notably, it is abolished by a mutation in gap junctions in projection neurons and is found to be mediated by electrical synapses between excitatory local interneurons and projection neurons. As a behavioral consequence, we demonstrate that virgin females in the presence of vinegar become receptive more rapidly to courting males, while male courtship is not affected. Altogether, our results suggest that lateral excitation via gap junctions modulates odor tuning in the antennal lobe and drives synergistic interactions between two ecologically relevant odors, representing food and sex.

sex pheromone | mixture synergism | functional imaging | electrical synapse | courtship behavior

Synergism can be defined as the cooperation of two or more elements operating together to achieve an effect that is greater than the sum of the individual effects. It is a ubiquitous and crucial aspect of nature and has provided a functional basis for the evolution of complex systems (1). It has been observed, for example, that synergistic interactions between multilevel, multimodal circuits enhance selection for the fastest mode of escape behavior in *Drosophila melanogaster* (2). In the same way, synergistic effects between plant-emitted volatiles and specific aromatic compounds are known to modulate attraction behavior of several insect species (3–5). Like plant volatiles, animal-produced sex pheromones interact with habitats and food signals to enhance an animal's behavioral acuity (6, 7). Although such interaction between two chemosensory cues—namely, food and sex—is known to drive reproductive isolation and speciation (8, 9), the underlying neuronal mechanism has so far remained elusive. Hence, in this study, we aim to unravel the neural circuitry that leads to synergism between food and sex odors in *D. melanogaster*.

Most insects, including the vinegar fly *D. melanogaster*, heavily depend on their olfactory system when they perform elementary activities, such as feeding, mating, ovipositing, and avoiding predators. During mating, the sex pheromone *cis*-vaccenyl acetate (cVA), produced by males, plays a significant and sex-specific role in communication between males and females. Whereas cVA evokes aggressive behavior in males and suppresses courtship with other males (10, 11), it increases sexual receptivity in females (10). cVA perfuming of miR-124 mutants males, which generally produce less cVA, restored their ability to achieve copulation with females (12). cVA also acts as an aggregation-promoting pheromone, attracting both males and females to food (13–16). In nature, odors do not usually occur as single cues but, rather, are perceived as a blend, consisting of different odor components. Vinegar flies mostly aggregate, oviposit (17), and mate (18) on fermenting fruits. As pheromone communication and food odor reception naturally occur together, we hypothesized that these odors are also linked at the neuronal level. Recently, it has been shown that virgin fed *Drosophila* females are more attracted to the blend of cVA and vinegar than to vinegar alone in different behavioral assays, while males are not (19). Vinegar represents a complex blend and highly attractive food odor to *D. melanogaster* (20). Insulin signaling was reported to partially control cVA perception (depending on a fly's nutritional state) and to modulate sexual receptivity in virgin females (19).

The architecture of the *Drosophila* olfactory circuit has been nearly fully characterized. The antenna houses ∼40 different types of olfactory receptors (ORs), which are expressed in olfactory receptor neurons (ORNs). ORNs expressing the same ORs project onto the same glomerulus in the antennal lobe (AL) (21), the primary olfactory center of the fly brain. Furthermore, ORNs expressing the same ORs exhibit the same odor response properties (22). In each glomerulus, the axons of the ORNs synapse onto the dendrites of the corresponding projection neurons (PNs) (23, 24). In adult male and female flies, the sex pheromone cVA is perceived by ORNs expressing OR67d, and

Significance

We elucidated the neuronal mechanism underlying the interaction of two ecologically relevant odors in the fly brain. Our study demonstrates that exposure to the male-produced sex pheromone *cis*-vaccenyl acetate, in combination with the complex food odor vinegar, evokes an enhanced and synergistic functional response in the primary olfactory center of virgin female flies. This effect arises within the neuronal network of the antennal lobe and is mediated by electrical synapses. The synergistic response in virgin females leads to an increased sensitivity to the pheromone, and therefore an enhanced female receptivity during courtship. This mechanism is highly useful, since it promotes mating in females when food is present: that is, when the nutritional supply of the female and its offspring is guaranteed.

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these ORNs project onto the DA1 glomerulus in the AL (10). Gender-specific differences in behavioral response to cVA, which derive from sexually dimorphic third-order olfactory neurons, have been observed (25–28). In addition, gender-specific differences in response to food odors have also been reported: the ionotropic receptor IR84a in *Drosophila* detects food odors, such as phenyl acetic acid and phenyl acetaldehyde, and increases male courtship behavior without altering female receptivity (29). In addition, it has been recently shown that yeast increases the female's sexual receptivity through the interaction between its odorous fermentation product acetic acid, sensed by IR75a, and its nutritional content (30). Hence, by coupling the perception of food odors with the activation of the courtship circuitry, the specific sensory pathways coordinate both feeding and reproductive behaviors. However, how and where the different olfactory inputs interact has so far remained unknown. Therefore, in this study, we investigated the neuronal circuitry underlying the interaction between a sex pheromone and food odors in the *Drosophila* brain.

Results

The Food Odor Vinegar Enhances the Pheromone Response in the Glomerulus DA1. Vinegar in the presence of cVA has been reported to attract more virgin females than vinegar alone (19), suggesting that the perception of pheromone and food signals are modulated simultaneously. To scrutinize whether vinegar, a complex food odor (31), modulates the reception of the sex pheromone cVA, which is produced by males, we first focused on the primary olfactory center, the AL, and analyzed *Drosophila*'s functional response to the pheromone at the PN level. We performed functional imaging experiments using transgenic flies that genetically express the calcium sensor GCaMP3 under the control of the *GH146-Gal4* driver line to selectively monitor odor responses in uniglomerular PNs (Fig. 1*A*). We analyzed the odor-evoked responses of the cVA-responsive glomerulus DA1 during stimulation to cVA and vinegar, as well as the binary mixture of both at three different concentrations (10^{-3}, 10^{-2}, and 10^{-1}) (Fig. 1 *B* and *C*). As expected, cVA evoked a strong and clear response in the DA1 glomerulus in a dose-dependent manner, whereas vinegar did not elicit any activity in this glomerulus. Interestingly, in virgin females, the binary mixture of cVA and vinegar elicited a significantly higher response than cVA alone at concentrations of 10^{-2} and 10^{-1} (Fig. 1*C*). To examine whether the observed enhanced response is the result of an additive response of either odors or whether it represents the result of their interaction, we compared the sum of the individual responses to the measured mixture response (Fig. S1*A*). Since the measured response in the glomerulus DA1 to the binary mixture was significantly higher than the predicted additive response of both odors, the enhancement of the response we observed can be defined as synergism. Notably, we did not observe this synergistic effect in the glomerulus DA1 in virgin males (Fig. 1 *D*–*F* and Fig. S1*B*), which supports previous observations that behavioral interactions of cVA and food odors are restricted to females only (19). Interestingly, mated females also failed to show this phenomenon, since the response to the binary mixture equals the responses to the pheromone alone (Fig. 1 *G*–*I* and Fig. S1*C*). Moreover, the general PN response to cVA was very low in mated females compared with virgin females, which is well in line with previous results (32). To analyze whether the presence of vinegar enhances the sensitivity of virgin females to cVA in general, we established a dose–response curve to cVA at different concentrations against the background of a steady vinegar concentration (Fig. S1*D*). We observed that vinegar increases the sensitivity of virgin females to cVA in a ratio-dependent manner, meaning that only the 1:1 mixture induced a synergistic response. When we compare the responses between the binary mixture and the individual compounds across many glomeruli, we see that this synergistic effect occurs only in the pheromone-responsive glomerulus DA1 (all other glomeruli labeled by *GH146-Gal4* responded as predicted and did not show any kind of interactions) (Fig. S2). Unfortunately, the most responsive glomeruli to vinegar (i.e., glomeruli DL2d/v, DP1l, DC4) could not be monitored, since they were not labeled by *GH146-Gal4*.

To analyze whether the observed synergism is confined to the mixture of vinegar and cVA or whether it can be evoked by other combinations of odors, we measured the response of glomerulus DA1 to limonene [an oviposition cue (33)], to 1-hexanol (a neutral odor), to acetic acid [the major component of vinegar (34)], and to their binary mixture with cVA. However, neither limonene nor 1-hexanol elicited a significant increase in the DA1 response when presented along with cVA, compared with when presented alone (Fig. 1 *J* and *K*). Interestingly, acetic acid, the main volatile component of vinegar, did not evoke any synergism in combination with cVA (Fig. 1 *J* and *K*), although it elicits behavioral attraction as a single compound (34). However, as Becher et al. (34) also observed, acetic acid alone does not nearly evoke the same grade of attraction as vinegar. It is therefore likely that the complete vinegar blend is necessary to elicit mixture synergism in combination with cVA and not just a single compound. Taking these data together, we find that the synergistic response of the glomerulus DA1 can be said to occur only in virgin females, in an odorant-specific and glomerulus-selective manner.

Synergism Between Pheromone and Vinegar Does Not Occur at the Sensory Level. We next wondered whether the synergism evolves at and derives from the peripheral level, and therefore performed extracellular single sensillum recordings (SSRs). As synergism was observed only in the cVA-responsive DA1 glomerulus, we limited our recordings to the at1 sensillum, which houses OR67d expressing ORNs. We examined the responses in virgin females to cVA, vinegar, and the binary mixture of both, again at three different concentrations. As expected, the OR67d-expressing ORNs responded specifically to cVA in a dose-dependent manner, but these ORNs did not show any response to vinegar alone (Fig. 2*A*). However, unlike the PNs, OR67d-expressing ORNs in virgin females did not show any enhanced response to the blend of cVA and vinegar (Fig. 2*A*). SSR data from the male at1 sensillum exhibited similar properties, wherein the response to the binary mixture revealed the same spike frequency as the response to cVA alone (Fig. S3*A*). We further performed functional imaging of ORNs in the AL by expressing GCaMP3 under the control of the *Orco* promoter (Fig. 2*B*). Because Orco expression is very heterogeneous in the different sensilla classes, and in particular low in trichoid sensilla (35), GCaMP expression in the AL varies accordingly. Hence, calcium signals in glomerulus DA1 are less sensitive compared with SSR and showed a clear calcium response to cVA only at a concentration of 10^{-1}. In accordance with the SSR data, neither the calcium responses in the female nor those in the male AL revealed any synergistic effect in the glomerulus DA1 to the mixture of cVA and vinegar (Fig. 2 *C* and *D* and Fig. S3 *B* and *C*). In addition, we performed optical imaging from IR8a-expressing ORNs in different vinegar-responsive glomeruli in virgin females, as vinegar activates strongly some glomeruli (e.g., DP1m, DP1l, DL2d/v, and DC4) innervated by ionotropic receptors (IRs) (36) (Fig. 2 *E* and *F*). Still we did not observe any synergistic responses to the mixture in those glomeruli. The mixture response was always equal to the response to the stronger component, which was vinegar in this case (Fig. 2*G*). Taken together, these results demonstrate that the observed synergism does not occur at the sensory level and therefore likely emerges within the neuronal network of the AL.

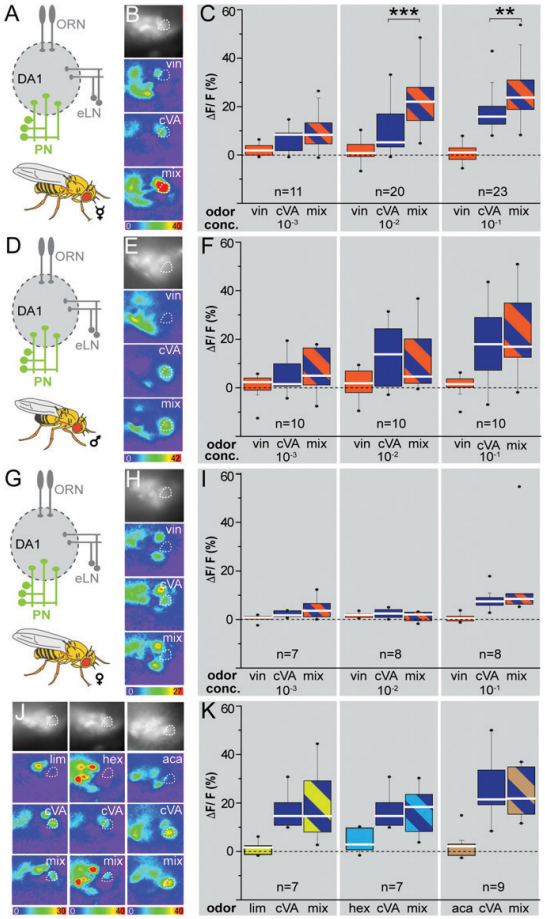

Fig. 1. PNs in the glomerulus DA1 reveal synergistic responses to the mixture of cVA and vinegar specifically in virgin females. (*A*) Schematic of the experimental approach: *UAS-GCaMP3* was expressed in PNs (green) using *GH146-Gal4* in virgin female flies. (*B*) Representative odor-evoked calcium responses of PNs in the AL of a virgin female to cVA, vinegar, and their binary mixture (10^{-1} concentration). (*C*) Box plots display $\Delta F/F$ responses in glomerulus DA1 in virgin females to vinegar (orange), cVA (blue), and their binary mixture (striped) at three different concentrations. The white line in the box represents the median. The mixture evokes a significantly enhanced response (***$P < 0.001$; **$P < 0.01$; Wilcoxon matched paired test). (*D*) Schematic of the experimental approach: *UAS-GCaMP3* was expressed in PNs (green) using *GH146-Gal4* in virgin male flies. (*E*) Representative odor-evoked calcium responses of PNs in the AL of a virgin male to cVA, vinegar, and their binary mixture (10^{-1} concentration). (*F*) Box plots display $\Delta F/F$ in DA1 in virgin males to vinegar (orange), cVA (blue), and their mixture (striped) at three different concentrations. The mixture evokes a similar response as cVA ($P > 0.05$; Wilcoxon matched paired test). (*G*) Schematic of the experimental approach: *UAS-GCaMP3* was expressed in PNs (green) using *GH146-Gal4* in mated female flies. (*H*) Representative odor-evoked calcium responses of PNs in the AL of a mated female to cVA, vinegar, and their mixture (10^{-1} concentration). (*I*) Box plots display $\Delta F/F$ in DA1 in mated females to vinegar (orange), cVA (blue), and their mixture (striped) at three different concentrations. The mixture evokes a similar response as cVA ($P > 0.05$; Wilcoxon matched paired test). (*J*) Representative odor-evoked calcium responses of PNs in the AL of a virgin female to limonene (lim), 1-hexanol (hex), acetic acid (aca), and their individual binary mixtures with cVA (10^{-1} concentration). (*K*) Box plots represent $\Delta F/F$ responses of PNs in DA1 to limonene (lim, yellow), 1-hexanol (hex, indigo), acetic acid (aca, brown), and cVA (blue), and the mixtures of cVA with the individual odors (striped boxes) at 10^{-1} concentration. None of the mixtures evokes a synergistic response ($P > 0.05$; Wilcoxon matched paired test). (Magnification in *B*, *E*, *H*, and *J*, 200×.)

Glomerulus DA1 Receives Input from Vinegar-Responsive ORNs Through Excitatory Local Interneurons. To pinpoint the origin of the synergistic effect, we proceeded to the next processing level along the olfactory pathways and examined the response to the mixture in local interneurons (LNs). As vinegar and the pheromone together induce a positive synergistic effect, we focused our interest on the population of excitatory LNs (eLNs). For this purpose, we expressed GCaMP3 using the enhancer trap line *Krasavietz-Gal4* and performed functional imaging of the AL (Fig. 3*A*). The majority of local interneurons, labeled by *Krasavietz-Gal4*, are excitatory in nature and coupled to other neurons through electrical synapses (37–39); they possess reciprocal synapses with PNs, inhibitory LNs (iLNs), and other eLNs, and transmit both depolarization and hyperpolarization, while chemical neurotransmission does not occur (38, 40). We analyzed the calcium responses of Krasavietz-positive eLNs in the glomerulus DA1 to cVA and to vinegar, and to their binary mixture at three different concentrations (Fig. 3*B*). We observed that, whereas these eLNs responded only minimally to all three odorants at the two lower concentrations (10^{-3} and 10^{-2}), they responded clearly and strongly to odorants at the highest concentration (i.e., 10^{-1}). Because LNs are multiglomerular in nature, eLNs in the DA1 glomerulus responded to both vinegar and to cVA. Interestingly, the binary mixture induced a significant stronger response compared with the response to the major component (i.e., here, vinegar) (Fig. 3*B*). However, as the measured response to the mixture was not significantly different compared with the predicted additive response to vinegar and cVA, this effect cannot be termed as synergism (Fig. S4*A*). In addition, we also measured the double concentration of vinegar, since the expected response to an odor mixture in the absence of interactions should not exceed the response to the double concentration of the stronger odor component (41). However, the response in glomerulus DA1 to the double vinegar concentration was equal to the measured mixture response (Fig. S4*A*), confirming that no synergistic response can be observed in eLNs.

Although no interaction takes place at the eLN level, it is still conceivable that these neurons are involved in initiating synergism to the mixture by conveying the input from ORNs responsive to food odors to the DA1 glomerulus, where the interaction takes place. Since Krasavietz-positive eLNs have been described as multiglomerular neurons (37, 42), these neurons should connect the pheromone glomerulus with the vinegar-responsive glomeruli and hence facilitate cross-talk at the AL level. To verify such a connection, we expressed photoactivatable GFP (*UAS-C3PA*) under control of the *Krasavietz-Gal4* driver line and photoactivated glomerulus DA1 to monitor the eLN processes from DA1 to other glomeruli throughout the whole AL (Fig. S4*B*). After the photoactivatable GFP diffused to other glomeruli, we quantified the intensity of those glomeruli that are responsive to vinegar before and after photoactivation (Fig. S4*C*). The observed significant increase in intensity in those glomeruli confirms that the Krasavietz-positive eLNs are connecting the glomerulus DA1 to other vinegar-specific glomeruli. Hence, it is conceivable that eLNs spread and transmit the olfactory input from vinegar-specific glomeruli to the cVA-specific glomerulus DA1, leading to a subsequent synergistic response in the downstream neurons (i.e., in PNs).

Next, we wondered why the mixture synergism could only be observed for the vinegar-cVA mixture but not for other odors blended with cVA, because eLNs are innervating the majority of glomeruli (37, 42). We therefore performed imaging from

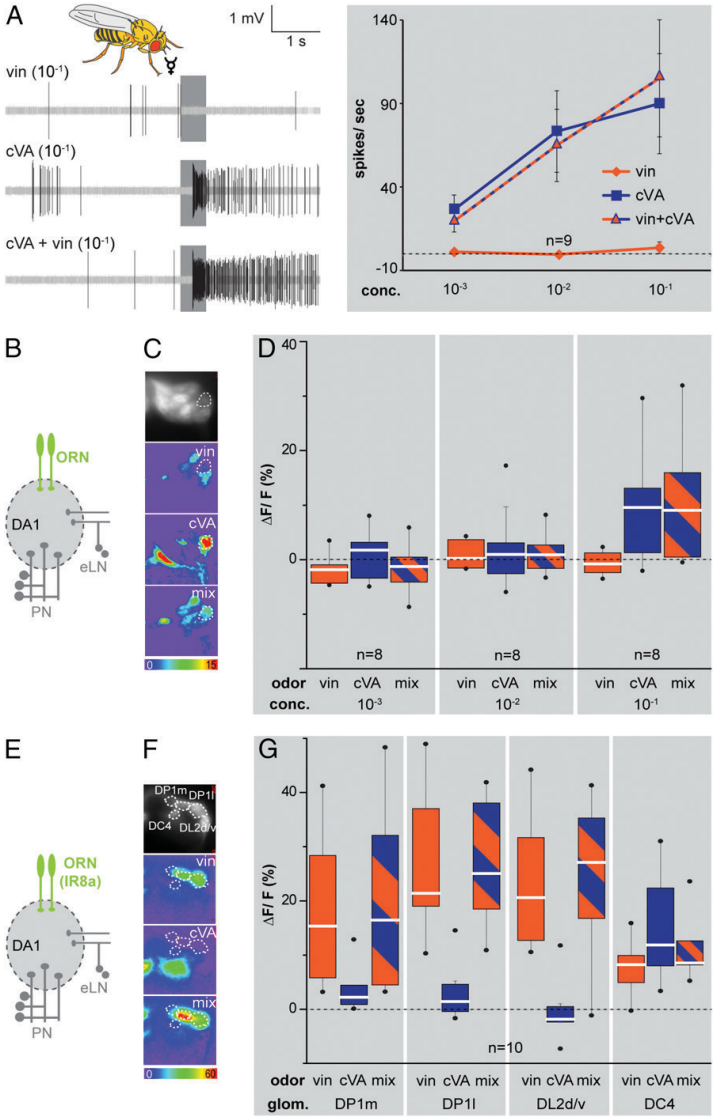

Fig. 2. Mixture synergism does not occur at the sensory level. In vivo extracellular SSRs from the at1 sensillum expressing OR67d. (*A*, *Left*) Representative traces display the response of OR67d ORNs in virgin females to vinegar, cVA and their binary mixture (10^{-1} concentration). (*Right*) Line curves represent the averaged neuronal activity (spikes per second) to vinegar (orange), cVA (blue), and their binary mixture (striped) at three different concentrations ($P > 0.05$; Wilcoxon matched paired test). (*B*) Schematic of the experimental approach: *UAS-GCaMP3* was expressed in the majority of ORNs (green) using *Orco-Gal4* in virgin females. (*C*) Representative odor-evoked calcium responses of ORNs in the AL of a virgin female to cVA, vinegar, and their binary mixture (10^{-1} concentration). (*D*) Box plots represent $\Delta F/F$ responses of ORNs in the glomerulus DA1 in virgin females to vinegar (orange), cVA (blue), and their binary mixture (striped boxes). The white line in the box represents the median. The ORN response to the mixture is equal to the response to the stronger component (i.e., cVA) ($P > 0.05$; Wilcoxon matched paired test). (*E*) Schematic of the experimental approach: *UAS-GCaMP3* was expressed in ORNs expressing IRs (green) using *IR8a-Gal4* in virgin females. (*F*) Representative odor-evoked calcium responses of IR8a-expressing ORNs in the AL of a virgin female to cVA, vinegar, and their binary mixture (10^{-1} concentration). (*G*) Box plots represent $\Delta F/F$ responses of IR8a-expressing ORNs in different vinegar-responsive glomeruli in virgin females to vinegar (orange), cVA (blue), and their binary mixture (striped boxes) at 10^{-1} concentration. The ORN response to the mixture is equal to the response to the stronger component (i.e., vinegar) ($P > 0.05$; Wilcoxon matched paired test). (Magnification in *C* and *F*, 200×.)

Krasavietz-positive eLNs to vinegar and two other previously used odors, 1-hexanol and limonene, to investigate whether they differentially activate glomerulus DA1. Notably, these two odors did not induce any synergistic mixture response when combined with the pheromone cVA (Fig. 1*K*). Indeed, the quantification of the eLN response reveals that vinegar induced

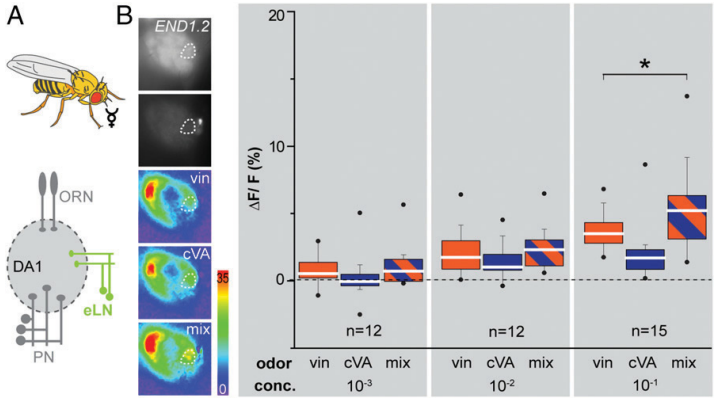

Fig. 3. Excitatory local interneurons do not reveal a synergistic mixture response. (*A*) Schematic of the experimental approach: *UAS-GCaMP3* was expressed in eLNs (green) using *Krasavietz-Gal4* in virgin female flies. (*B*, *Left*) Representative odor-evoked calcium responses of eLNs in the AL in the background of *END1-2* (elav-n-synaptobrevin:DsRed) of a virgin female to cVA, vinegar, and their binary mixture (10^{-1} concentration). (*Right*) Box plots display $\Delta F/F$ responses in the glomerulus DA1 in virgin females to vinegar (orange), cVA (blue), and their binary mixture (striped) at three different concentrations. The white line in the box represents the median. The eLN response to the mixture is significantly higher than the response to the stronger component (i.e., vinegar) at 10^{-1} concentration. ($P < 0.05$; Wilcoxon matched paired test). *$P = 0.03$. (Magnification in *B*, 200×.)

a significantly stronger activity in glomerulus DA1 than the other two odors (Fig. S4*D*). This result is in line with previously published electrophysiological recordings of Krasavietz-positive eLNs, demonstrating that they exhibit distinct odor response patterns (38). Hence, irrespective of the multiglomerular morphology of eLNs, their selective odor responses might drive the vinegar-specific synergism in glomerulus DA1.

Electrical Synapses Between eLNs and PNs Mediate Synergism. As mentioned above, eLNs are largely connected to PNs through gap junctions (38, 40). To investigate whether the synaptic connections between eLNs and PNs actually mediate the interaction between the two odors, vinegar and cVA, we analyzed whether the mixture-induced synergism in PNs is evident in flies with mutated gap junctions. In invertebrates, gap junctions are composed of intercellular channels formed by innexin proteins. Among eight types of innexins in *Drosophila*, *shakB* (inx8) is expressed in scattered neurons, the giant fiber neural pathway, and the AL (40, 43, 44). The *shakB*^{2} mutant exhibits disrupted electrical connections in the optic lobe and in the giant fiber escape pathway (45, 46). In the *Drosophila* AL, four kinds of synapses possess gap junctions and are therefore affected by the *shakB*^{2} mutation: eLNs-to-PNs, PNs-to-PNs, eLNs-to-iLNs, and eLNs-to-eLNs (38, 40). Hence, the olfactory input to the AL should function normally in the *shakB*^{2} mutant fly, while the synaptic transmission of eLNs should be disrupted. Notably, functional imaging from PNs in the *shakB*^{2} mutant background did not reveal any synergism in the glomerulus DA1 (Fig. 4 *A* and *B*), indicating that gap junctions are necessary to drive the synergism in the pheromone glomerulus that is induced by the exposure to both cVA and vinegar. As the *shakB*^{2} mutation causes a global loss of electrical synapses, which is not limited to the AL, we next used an RNAi construct against inx8 (i.e., RNAi of *shakB*) (47) to block gap junctions in olfactory PNs. To achieve this, we expressed *inx8-RNAi* in PNs of *GH146-Gal4* and monitored their response to the mixture as well as to the individual odors via functional imaging at two concentrations, 10^{-2} and 10^{-1} (Fig. 4*C*). In line with our previous observation, these flies failed to show any mixture-induced synergistic response in PNs of the glomerulus DA1. It is important to note here that the enhancer trap line *GH146-Gal4* does not label solely PNs, but also a few additional higher-order neurons, such as a subset of Kenyon cells in the mushroom body, a small group of descending interneurons ventral to the lateral protocerebrum (48), and a GABAergic anterior paired lateral neuron innervating the mushroom body (49). We therefore cannot rule out the possibility that those neurons were also affected by silencing gap junctions, although the importance of electrical coupling for odor processing has so far been proven solely for the AL (40).

As gap junctions are bidirectional and require the ShakB protein at both the presynaptic and the postsynaptic sites to function properly (45), we next rescued the wild-type ShakB protein in both eLNs and PNs. For this purpose, we employed a transgenic fly as a control strain in which Krasavietz-positive eLNs and GH146-positive PNs expressed GCaMP6s (Fig. 4*D*). We first verified that the synergistic response to the mixture was visible when we recorded eLNs along with PNs, and performed imaging from both sets of neurons to vinegar, cVA, and their binary mixture. Indeed, these control animals also showed a significantly increased response to the binary mixture compared with their response to the individual odors at two concentrations, 10^{-2} and 10^{-1} (Fig. 4*E*). Confirming our previous results, the *shakB*^{2} mutation abolished the synergistic response in PNs and eLNs. By expressing and rescuing wild-type *shakB.neural* in eLNs and PNs in the background of the *shakB*^{2} mutation, we were able to restore the synergism to the mixtures (Fig. 4*E*).

Altogether, our observations suggest that gap junctions between eLNs and PNs, and within PNs, are necessary and sufficient to drive synergism in the glomerulus DA1 and therefore to enhance the response to cVA in the presence of the complex food odor vinegar.

Exposure to Vinegar Modulates Female Receptivity, Which Requires Gap Junctions in PNs. Our functional imaging results indicate that vinegar modulates the olfactory system of virgin females in a way that enhances their sensitivity to cVA. However, what does that mean for a female fly in nature? In female flies cVA governs both aggregation and mating. A previous study has shown that the mixture of vinegar and cVA becomes behaviorally more attractive to virgin females than vinegar alone (19), meaning that the aggregation-promoting response of flies to cVA is increased by vinegar. However, do food odors also influence mating

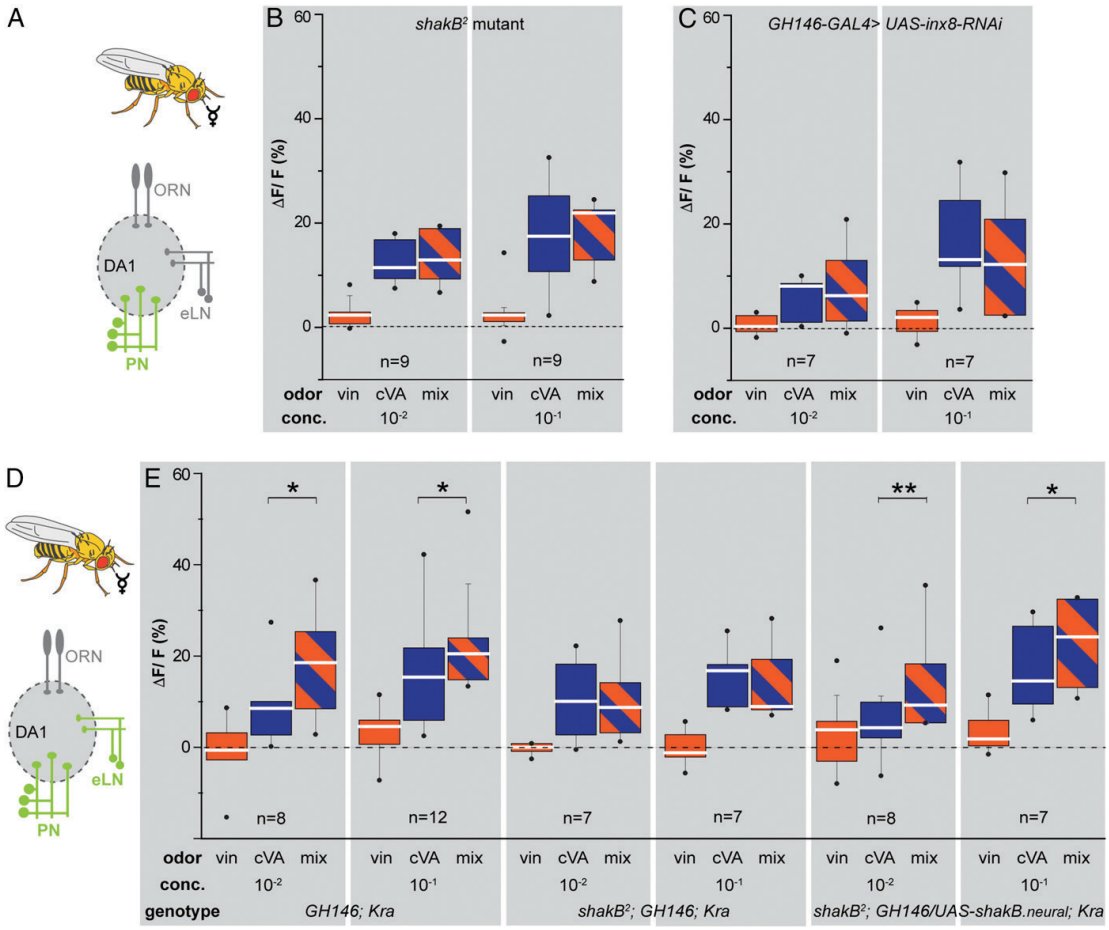

Fig. 4. Gap junctions between PNs and eLNs are necessary and sufficient to induce mixture synergism. (*A*) Schematic of the experimental approach: *UAS-GCaMP3* was expressed in PNs (green) using *GH146-Gal4* in virgin females. (*B*) Box plots display Δ*F*/*F* responses in the glomerulus DA1 in virgin females, in the background of the *shakB*^{2} mutant to vinegar (orange), cVA (blue), and their binary mixture (striped) at two different concentrations (10^{-2} and 10^{-1}). The white line in the box represents the median. The mixture does not evoke a synergistic response ($P > 0.05$; Wilcoxon matched paired test). (*C*) Box plots display Δ*F*/*F* responses in the glomerulus DA1 in virgin females to vinegar (orange), cVA (blue), and their binary mixture (striped) at two different concentrations (10^{-2} and 10^{-1}). Gap junctions have been blocked in PNs using RNAi against inx8. The mixture does not evoke a synergistic response ($P > 0.05$; Wilcoxon matched paired test). (*D*) Schematic of the experimental approach: *UAS-GCaMP6s* was expressed in PNs and eLNs (green) using *GH146-Gal4* and *Krasavietz-Gal4* in virgin females. (*E*) Box plots display Δ*F*/*F* responses in the glomerulus DA1 in virgin females to vinegar (orange), cVA (blue), and their binary mixture (striped) at 10^{-2} and 10^{-1} concentration. Genotypes are as follows: control line, *GH146-Gal4*; *Krasavietz-Gal4*; mutant line, *shakB*^{2}; *GH146-Gal4*; *Krasavietz-Gal4*; rescue line, *UAS- shakB.neural/GH146-Gal4*; *Krasavietz-Gal4* in the *shakB*^{2} mutant background. The control and rescue lines show a synergistic mixture response at both concentrations (*$P < 0.05$, **$P < 0.01$; Wilcoxon matched paired test).

behavior in flies? The presence of the food odors phenyl acetic acid and phenyl acetaldehyde increases courtship behavior in males via the IR84a-dependent pathway (29), although in females, mating behavior remains unaltered. Due to our findings of a synergism of cVA and vinegar, we asked whether the latter influences the courtship behavior of female flies. We therefore monitored the courtship behavior of a wild-type virgin male and female in a closed arena in the presence of either water or vinegar. Because the behavioral assay was performed in a closed small chamber for an extended period of time (20 min), all behavioral experiments were carried out with exposure to a low concentration of vinegar (i.e., 10^{-3}). Interestingly, while the copulation success of flies was not significantly affected by vinegar (Fig. 5*A*), flies mated significantly earlier in the presence of this food odor (Fig. 5*A′*). To analyze whether the female's receptivity or the male's perception was modulated by vinegar, we quantified the courtship index. However, the presence of vinegar does not affect the courtship index and therefore does not influence the male's courting behavior at all (Fig. 5*A″*), implying that only the female's receptivity is affected.

To verify whether vinegar modulates the female's receptivity only through the cVA pathway, we paired a *OR67d* mutant virgin female, which cannot detect cVA, with a wild-type virgin male in the courtship assay. As expected, only 21% of flies copulated in this experiment; this low percentage was shown previously to be due to the lack of pheromone perception (10) (Fig. 5*B*).

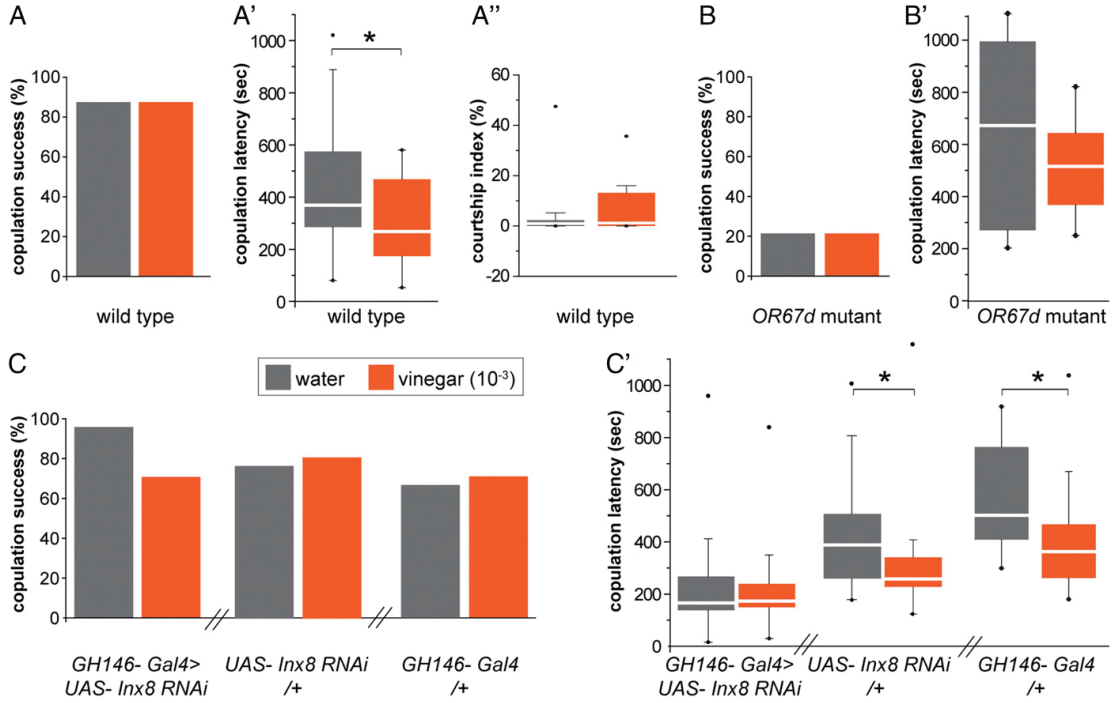

Fig. 5. Vinegar modulates copulation latency in females, which requires gap junctions in PNs. Courtship behavior assays performed with wild-type and different mutant flies in the presence of water (gray) or vinegar (10^{-3}, orange). (*A* and *A′*) Histograms represent copulation success and the box plots show the copulation latency of wild-type pairs of *D. melanogaster*. The presence of vinegar significantly reduces copulation latency, while copulation success is unaffected (*$P < 0.05$; Mann–Whitney test; $n = 24$). (*A″*) Box plots reveal courtship index of wild-type pairs. The presence of vinegar does not significantly affect the courtship index ($n = 12$). (*B* and *B′*) Histograms represent copulation success and the box plots show the copulation latency of wild-type males (*Canton-s*) and *OR67d* mutant females. Neither copulation success nor latency are influenced by the presence of vinegar ($P > 0.05$; Mann–Whitney test; $n = 24$). (*C* and *C′*) Histograms represent copulation success and the box plots show the copulation latency of wild-type males (*Canton-s*) and mutant females in which gap junctions have been blocked in PNs (*GH146-Gal4 > UAS-inx8-RNAi*), as well as the parental control lines (*UAS-inx8-RNAi/+* and *GH146-Gal4/+*). Only the parental lines show a reduced copulation latency in the presence of vinegar (*$P < 0.05$; Mann–Whitney test; n = 24). χ^2 Test with Yates correction was used for copulation success and Mann–Whitney test was used for copulation latency.

However, the copulation latency of female flies in this experiment did not differ significantly between flies exposed to water or to vinegar (Fig. 5*B′*), indicating that vinegar acts exclusively through the OR67d pathway.

Next, we were curious to know whether the change in receptivity mediated by vinegar depends on the gap junction at the eLN-PN level, as implied by our functional imaging experiments. For this purpose, we paired a wild-type virgin male with a mutant virgin female whose gap junctions in PNs had been blocked by expressing *inx8-RNAi* under control of the *GH146-Gal4* driver line. Notably, we did not observe any significant difference regarding either copulation success or copulation latency in flies exposed to water or vinegar (Fig. 5 *C* and *C′*), indicating that the vinegar-induced reduction in receptivity depends on electrical synapses in PNs. As predicted, the parental controls (i.e., a *UAS-inx8-RNAi*/+ or *GH146-Gal4*/+ female paired with a wild-type male, respectively) became receptive more rapidly in the presence of vinegar while the level of copulation success remained similar to that observed in wild-type flies (Fig. 5 *C* and *C′*). Taken together, our results demonstrate that vinegar modulates and increases the female flies' sensitivity to cVA, by being mediated through electrical synapses at the eLN-PN level within the DA1 glomerulus (Fig. 6). Both odors, cVA and vinegar, activate glomerulus DA1 through two different pathways: while cVA directly activates glomerulus DA1 through OR67d-expressing ORNs, vinegar indirectly enhances the DA1 activation via lateral excitation by eLNs. At a later stage, the two different pathways converge at the output level of the AL and lead to a subsequent synergistic mixture response in glomerulus DA1 at the PN level. As a behavioral consequence, this modulation causes the virgin female to become receptive more rapidly to courting males.

Discussion

Interaction Between Food Odors and Sex Pheromone. In nature, odors always occur as blends, and each odor component may affect the perception of another odor. In the context of *Drosophila*, as flies always feed, mate, and oviposit on fermenting food, food odors are part of an ever-present, unavoidable background of every odor that flies encounter, such as aggregation cues, male-emitted sex pheromones, parasitoid odors (50), or oviposition cues (33). It is evident that a specific class of ionotropic receptors in *Drosophila*, namely IR84a, is activated not by fly-derived chemicals (the volatile sex pheromones) but by the compounds present in food which promote courtship behavior in males (29). Food odors are also reported to enhance the attraction of female *Drosophila* to the male-emitted cVA

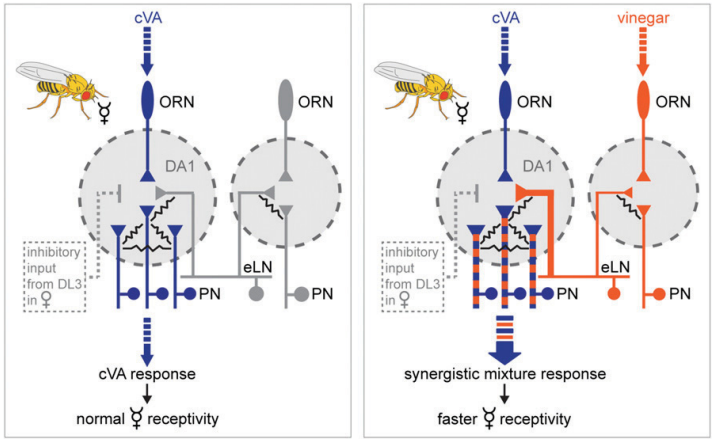

Fig. 6. Circuit model for mixture synergism. Proposed mechanism underlying the observed synergism in virgin females to the mixture of the sex pheromone cVA and the complex food odor vinegar. (*Left*) The sole cVA stimulation, which is detected by ORNs expressing OR67d that target glomerulus DA1 in the AL. As a result, PNs in glomerulus DA1 are activated, which transfer the cVA response to higher brain centers promoting courtship and virgin female receptivity. (*Right*) Illustration of how the simultaneous stimulation with vinegar and cVA enhances the activity of DA1 in a synergistic manner. Vinegar activates specific vinegar-responsive glomeruli which convey this input through eLNs to the DA1 and other glomeruli via electrical synapses. Since DA1 receives a stronger lateral excitation by vinegar (thick line) than other glomeruli (thin line), the PNs of DA1 are stronger activated. As glomerulus DA1 possesses a large number of electrically coupled sister PNs, the signal gets further amplified and leads to the observed synergistic mixture response. The resultant synergistic activity of DA1 is reflected behaviorally by a faster receptivity of virgin females to courting males in the presence of vinegar. As previously shown, in the mated female glomerulus DL3 suppresses the cVA response in glomerulus DA1 via inhibitory LNs; as a result, the synergism cannot occur in this scenario (32).

depending on their nutritional state (19), further supporting the fact that food odors interact with pheromone perception.

In our study, we have identified and characterized the neuronal mechanism underlying the interaction of exposure to the complex food odor vinegar and to the male-specific sex pheromone cVA at the primary olfactory circuit level. We demonstrated that exposure to vinegar synergistically enhanced the flies' response to cVA in PNs in a glomerulus-specific and odorant-selective manner. Moreover, we were able to show that this synergistic response is mediated through electrical synapses between eLNs and PNs in the fly AL. The food odor in this case enhanced the virgin female's sensitivity to cVA. As mentioned above, a similar influence of other food odors (phenyl acetic acid and phenyl acetaldehyde) on male courtship through IR84a has been reported (29). In their study, the food odor affected the behavior of males only. In our study, a different food odor modulated the response of virgin females to cVA without having an effect on males, indicating that environmental cues affect males and females differentially through separate neuronal mechanisms. Although there is evidence that odorant interactions take place at the level of ORNs (51–53), we did not observe any synergistic effect at the peripheral site. Vinegar is a complex blend of odors, where individual components activate different sets of ORs and IRs. Acetic acid alone, in combination with cVA, fails to evoke any synergism, suggesting that the complete vinegar blend is necessary to mediate a synergistic mixture response in PNs. It is conceivable that eLNs need to be activated in an optimum or strong level to achieve this synergism. Hence, the presence of all components of vinegar and consequently the activation of a specific OR/IR combination might be crucial, and need to be elucidated in further studies.

Different Aspects of Synergism. Can we term our observed mixture effect synergism, although vinegar does not directly activate glomerulus DA1? To induce a synergistic response, both stimulations do not necessarily need to share the same input pathways as, for example, demonstrated for synergistic interactions between different sensory modalities (2). Nociceptive and mechanosensory stimulations have been shown to lead to a synergistic behavioral output mediated by two different neuronal pathways that converge at a late stage of the sensory processing hierarchy. In our case, the direct cVA-mediated activation of glomerulus DA1 converges with an indirect lateral excitation induced by vinegar resulting in a synergistic glomerular activation at the AL output level and an enhanced behavioral output.

As already mentioned earlier, we wondered why the synergism via electrical synapses is confined only to vinegar and does not occur with other odor mixtures, since the Krasavietz-positive eLNs are multiglomerular and should therefore be activated also by other odors. We think the synergistic effect evoked by vinegar can be explained by the functional properties of these eLNs. The Krasavietz-positive eLNs have been shown to respond selectively to odor stimuli pronounced by their distinct firing patterns to different odors, while each odor elicited distinct responses in different Krasavietz-positive eLNs (38). Notably, this property is in contrast to the similar responses of inhibitory LNs to distinct odors (54, 55). According to our observation, vinegar activates the eLNs in glomerulus DA1 stronger than other odors, which in turn leads to a stronger activation of PNs in DA1 mediated by the eLNs-PNs gap junctions. This selective odor-response property of eLNs provides the basis for driving synergistic interaction in a glomerulus- and odor-specific manner.

Notably, we observed the synergistic effect of exposure to vinegar on courtship latency only in virgin females, while mated females failed to show this effect (Fig. S1*C*). This feature brings the plastic nature of the synergistic effect as the change in physiological state of the animal modulates the observed phenotype. The difference might be due to the chronic exposure to a high amount of cVA during mating, which activates the olfactory receptor OR65a targeting the DL3 glomerulus (32). OR65a ORNs decrease the activity of the DA1 glomerulus, most likely via inhibitory LNs. Decreased activity in DA1 results in an inhibition of cVA attraction behaviorally (32). Due to this inhibition onto glomerulus DA1, it is likely

that vinegar fails to enhance the activity of this glomerulus in combination with cVA, resulting in the absence of synergism.

Interestingly, virgin males also did not show any enhanced attraction to the odorant mixture in behavioral assays (19), which is well correlated to our observations derived from the functional imaging of AL PNs in males (Fig. 1). Although the branching patterns of cVA-specific PNs originating from the glomerulus DA1 differ in a gender-specific manner in their target area (i.e., the lateral horn) (25, 27), so far there is no evidence for any sex-specific innervation pattern at the level of the AL. However, although there seems to be no anatomical difference at the PN level of the glomerulus DA1, a sexually dimorphic response pattern has been reported: in males, PNs innervating DA1 responded preferably and more strongly to an ipsilateral cVA stimulus, whereas in females, PNs responded equally to both an ipsilateral and a contralateral stimulus (56). Whether this difference in the response pattern seen in glomerulus DA1 between males and females is in any way related to our observed sex-specific synergism needs to be addressed in further studies. In addition, it is conceivable that the innervation patterns of LNs is gender-specific in the pheromone-responsive glomeruli and could therefore lead to differential lateral processing between males and females. This assumption needs to be tested in future studies.

Functional Significance of Gap Junctions for Odor Tuning. We demonstrate here that exposure to vinegar enhances the fly's response to the sex pheromone cVA at the PN level. Although the population of eLNs does not show any synergistic response to the mixture, those neurons are necessary to initiate and mediate the synergism. It has been shown that eLNs significantly mediate lateral excitation in the AL (37, 39), and therefore they most likely convey the excitatory input from vinegar-responsive glomeruli to the DA1 glomerulus. eLNs labeled by the *Krasavitz-Gal4* line are connected to GH146-positive PNs only through reciprocal gap junctions (40), and the eLN-to-PN connection has a stronger impact than vice versa (40). The *Krasavietz-Gal4* line could be classified into two different LN subpopulations, namely type I and type II, based on their physiological properties and glomerular innervation patterns (38, 42). Among them only type I is coupled to other AL neurons via gap junctions. In addition, according to Huang et al. (38), type II Krasavietz neurons are probably inhibitory LNs. Since rescuing wild-type *shakB.neural* in Krasavietz-positive eLNs and -PNs rescued the mixture synergism, it is most likely that the gap junctions between type I eLNs and PNs are necessary to mediate the synergistic effect.

As eLNs are electrically coupled to GH146-positive PNs in multiple glomeruli, the question arises: How is the observed synergism limited to the DA1 glomerulus and not found in other glomeruli? The strength of the connectivity of eLNs to PNs is largely variable across glomeruli, and eLNs have been shown to respond selectively to odor stimuli (38). In addition, the glomerulus DA1 possesses an unusually large number of sister PNs (seven to eight PNs) compared with other glomeruli in the AL (35, 40, 57). As a result, the probability that dense electrical coupling will evolve between eLNs and PNs is higher in the glomerulus DA1 than in more broadly tuned glomeruli, such as the vinegar-responsive ones. These factors may explain why synergism is restricted to the cVA-responsive DA1 glomerulus. However, other narrowly tuned glomeruli with high PN innervations (35) might be the site of additional synergistic interactions and should be studied in the future.

PNs in the glomerulus DA1 detect cVA through OR67d-expressing ORNs located on the antennae, whereas they receive the vinegar-evoked signal most likely through electrically coupled eLNs. In the DA1 glomerulus, PNs possess two kinds of electrical synapses: eLNs-to-PNs and PNs-to-PNs connections (40). Gap junctions represent sophisticated synapses because of their high transmission speed, bidirectionality, and analogical nature, meaning that they transmit graded (i.e., also subthreshold) excitations and inhibitions (40, 58). Hence, neurons that are electrically coupled detect and transmit coincident subthreshold depolarization, which in turn increases neuronal excitability and promotes the temporal synchronization of firing (59–61). In sensory systems, electrical synapses have been shown to mediate lateral excitation and thereby improve sensory sensitivity (62–64). Applied to our results, the synchronous firing of electrically coupled eLNs-to-PNs and PNs-to-PNs, deriving from cVA- and vinegar-responsive glomeruli, leads to an enhancement of *Drosophila's* sensitivity to cVA in the presence of vinegar, such as food. Because cVA acts as a mating cue for the female, the presence of food during courtship increases the sexual receptivity of the virgin female without affecting male courtship. From an ecological point of view, this mechanism sounds logical, since reproductive behavior depends highly on the nutritional state of the female fly (19). Hence, the herein described circuit promotes mating when food is present: that is, when the nutritional supply of the female and its offspring is guaranteed. Future studies will elucidate how this synergism involving food and pheromone is relayed to higher processing centers, and will investigate whether this neuronal mechanism applies to other concurrent sensory inputs.

Materials and Methods

Flies were raised on autoclaved cornmeal-yeast-sucrose-agar food in a 12-h light/dark cycle at 25 °C incubator. Newly emerged flies were anesthetized with CO_2, and virgin males and females were collected, kept in separate vials, and fed fresh food for 4–7 d. Following lines have been used for functional imaging, *Orco-Gal4*, *GH146-Gal4* (II) (48), *Krasavietz-Gal4* (III) (37, 39), *IR8a-Gal4* (II) (65), *GH146-QF*, *QUAS mtd Tomato*, *UAS-GCaMP3* (66), and *UAS-GCaMP6s* (67). The above-mentioned stocks were obtained from Bloomington *Drosophila* Stock Center. For gap junction mutation, $shakB^2$ (X) (40, 68) and *UAS-shakB.neural* (II) (40, 45) were obtained from Mani Ramaswami's laboratory (Department of Genetics, Trinity College Dublin, Dublin). For photoactivation experiments, *UAS-C3PA* (27) was used. *UAS-inx8 RNAi* was obtained from the Vienna *Drosophila* RNAi Center (VDRC); Canton-S, an OR67d knock-in mutant (10), obtained from the Research Institute of Molecular Pathology, was used for the behavioral experiments.

Details on optical imaging, data analysis, the photoactivation procedure, SSRs, and the behavioral assays are provided in *SI Materials and Methods*.

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Supporting Information

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SI Materials and Methods

Optical Imaging. Flies were dissected for optical imaging according to the protocol of Strutz et al. (69). Flies were briefly immobilized on ice and then mounted onto a custom-made stage. Protemp II composite (3M ESPE) was used to fix each head. We bent the anterior part of the fly's head with fine gold wire, and a small plastic plate having a round window was placed on top. We sealed the head with that plate using two-component silicone (Kwik Sil) and leaving the center part open to make a cut. The cuticle between the eyes and the ocelli was cut under saline (130 mM NaCl, 5 mM KCl, 2 mM $MgCl_2$, 2 mM $CaCl_2$, 36 mM saccharose, 5 mM Hepes, 1 M NaOH, pH 7.3). The cuticle was either bent forward and fixed to the silicon or removed. After cleaning the fatty tissues and trachea, we were able to visualize the antennal lobes.

We used a Till Photonic imaging system with an upright Olympus microscope (BX51WI) and a 20× Olympus objective (XLUM Plan FL 20×/0.95 W), as described in ref. 19 for the functional imaging. Among the odorants, cVA (from Pherobank) was diluted in mineral oil (Carl Roth) to make concentrations of 10^{-1}, 10^{-2}, and 10^{-3}, and balsamic vinegar was diluted in double-distilled water to make concentrations of 10^{-1}, 10^{-2}, and 10^{-3}. Six microliters of these dilutions were pipetted on a filter paper (~1 cm^2; Whatman), which was placed in Pasteur pipettes. For tests of odorant mixture, two filter papers, one containing cVA and one containing vinegar, were placed in the same pipette. We used filter papers with solvent alone as blanks. A stimulus controller (Stimulus Controller CS-55; Syntech) was used for odor application. Continuous airflow (1 L/min) and pulses of odor (0.1 L/min) were directed through an acrylic glass tube to the fly's antennae. Odor stimuli were injected into this airstream after 2 s for a duration of 2 s. The recording frequency during imaging was 4 Hz with 40 frames (i.e., 10 s) in total. Each odor was measured only once in each animal and the odor stimulation sequence was randomized for each experiment, while we always applied the odors with rising concentrations (i.e., from 10^{-3} over 10^{-2} to 10^{-1}). However, not all concentrations could always be measured in all animals. Therefore, the number of animals for each concentration might differ and is given in each plot. The interstimulus interval was at least 60 s to avoid any effects of adaptation or habituation. To test whether the odor responses, and in particular the mixture response, were reproducible from trial to trial, we measured repeated stimuli in single animals and observed that also three consecutive repetitions induced a significant synergistic mixture response.

Data Analysis. Further data were analyzed with custom-written IDL 6.4 software (ITT Visual Information Solutions). Manual movement correction and bleach correction were followed by the calculation of relative fluorescence changes ($\Delta F/F$) from the background. The glomeruli were identified according to ref. 70. The $\Delta F/F$ of all 40 frames was imported to an Excel file. The responses from frames 10–18 were averaged for the glomerulus of interest for all treatments. Wilcoxon matched paired test was used for all statistical analyses of the imaging data.

Photoactivation and Intensity Quantification. *UAS-C3PA* was driven under *Krasavietz-Gal4* in the background of *GH146-QF*, *QUAS mtd Tomato* for the photoactivation experiment. Four- to 5-d-old virgin females were dissected as described before. The photoactivation was performed on an MPCLSM (Zeiss LSM 710 NLO confocal microscope; Carl Zeiss) equipped with an infrared Chameleon Ultra diode-pumped laser (Coherent). An initial prephotoactivation scan of the whole antennal lobe was taken at 925 nm with 40× water immersion objective (W Plan-Apochromat 40×/1.0 DIC M27; Carl Zeiss). The DA1 glomerulus was identified based on a *GH146* projection pattern. A region of interest in the center of each DA1 glomerulus was photoactivated for ~10 min (2-min photoactivation followed by 2-min rest) using 760 nm of laser. We allowed 10–15 min for photoactivated GFP to diffuse in more distal neural processes. The postphotoactivation scan was taken using the same set-up as that used for the prephotoactivation scan. The average fluorescence intensity was measured using Fiji software. The average intensity was divided by the area of selection to obtain intensity per square micrometer. The intensity was calculated in the photoactivated DA1 glomerulus and vinegar-responsive glomeruli and compared with the before and after photoactivated brains. A Wilcoxon matched paired test was used for all statistical analyses.

Single Sensillum Recording. Four- to 6-d-old virgin flies were immobilized by wedging each into a pipette tip while fixing the protruding head with wax. The antenna was stabilized on a coverslip with a glass pipette between the second and third antennal segments. Tungsten electrodes were electrolytically sharpened by immersing them in a KNO_2 solution. The reference electrode was inserted into the eye of the fly. To measure the olfactory response to cVA, the recording electrode was placed into long trichoid sensilla, which were identified based on morphology and their characteristic odor response profile. Each time, the complete odor set including all concentrations was tested at one sensillum per fly. Changes in extracellular potentials were measured with the computer software Auto Spike 32 (v3.7). Signals were amplified 10× (Syntech Universal AC/DC probe), sampled with 10,666 Hz, and filtered (300–3 kHz with 50/60 Hz suppression). The stimulus controller Syntech IDAC-4 controlled and defined the properties of the odor puff. The pulse duration of the odor stimulation was 500 ms. Neuronal activity was recorded 3 s before and 10 s after pulse stimulation. A main and a pulse flow of 0.5 L/min were maintained.

Serial dilutions of 10^{-3}, 10^{-2}, and 10^{-1} (vol/vol) were made. While cVA was diluted in mineral oil, double-distilled water was used for balsamic vinegar. Solvents were also used as control stimuli. Pasteur pipettes containing two filter papers were used for odor stimulations. Filter papers were loaded with 6 μL of the aliquots.

To analyze the action potential frequency (spikes per second) over the total recording interval, a bin width of 25 ms was set. We quantified the physiological response of the odor stimuli by subtracting the calculated maximum frequency of 1 s before from 1 s after stimulus onset. The Wilcoxon matched paired test was used for all statistical analyses.

Behavior. Males and females were collected after eclosion and raised individually and in groups, respectively, for 4–6 d. For each experiment, typically, 24 courtship assays were performed in a (1-cm diameter × 0.5-cm depth) chamber covered with a plastic slide. The base of the chamber had a small pore in which 2 μL of vinegar (10^{-3}) or water (solvent control) was placed to perfume the chamber. Plastic mesh was placed underneath the mating chamber to restrict the flies' contact with vinegar or water. Courtship behaviors were recorded for 20 min and analyzed. All mating experiments were performed under red light (660-nm wavelength) at 25 °C and 70% humidity. Each video was ana-

lyzed for copulation success, which was measured by the percentage of males that copulated successfully in the first 20 min, and copulation latency, which was measured as the time taken by each male until copulation. For courtship experiments, females were decapitated with a clean razor blade, to avoid any successful mating during the courtship process. The assay was performed 20 min after decapitation. Courtship index was measured by a researcher who was blinded to genotype. Courtship index was calculated as the portion of time a male fly was engaged in any step of the courtship (chasing, orienting, wing vibration, abdominal curling, and copulation) in the first 10 min of the assay. The χ^2 test with Yates correction was used to statistically analyze the copulation success and the Mann–Whitney test was used for the copulation latency and the courtship index.

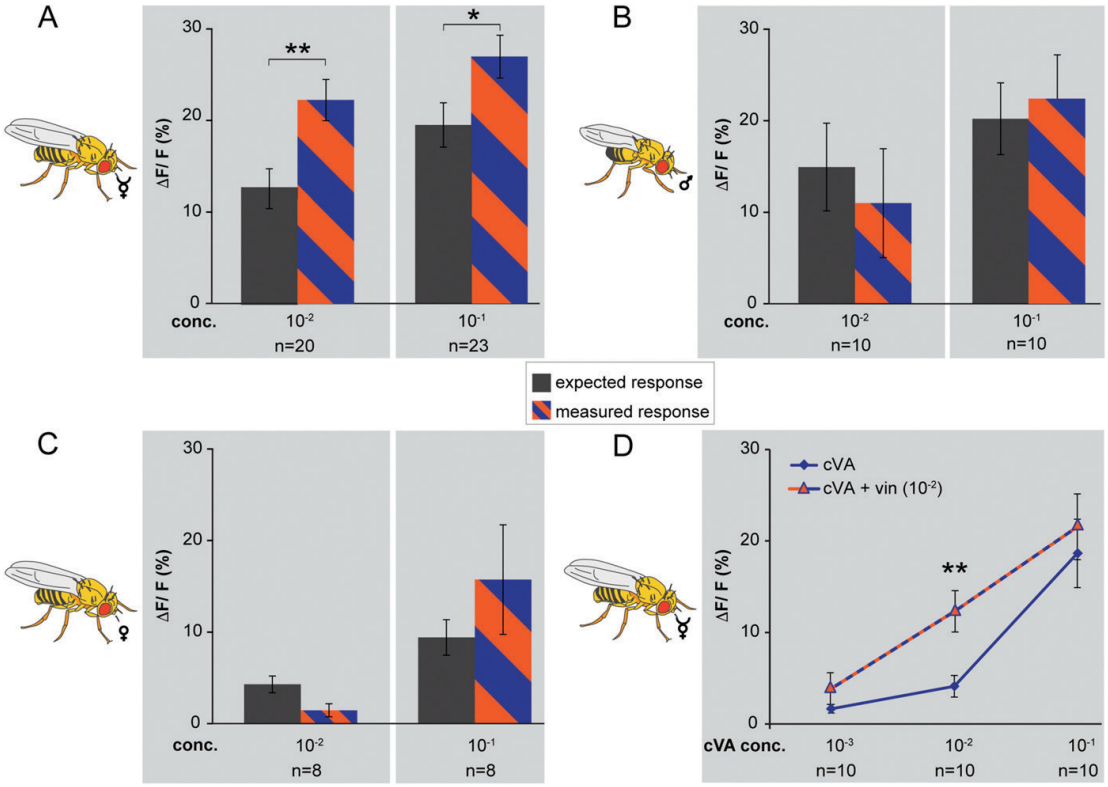

Fig. S1. PNs in glomerulus DA1 reveal synergism to the mixture of cVA and vinegar in virgin females, while males and mated females do not show any mixture interaction. (*A*–*C*) Comparison between expected (dark gray) and measured (striped) mixture response of PNs in glomerulus DA1 in virgin females (*A*), virgin males (*B*), and mated females (*C*) at 10^{-2} and 10^{-1} concentrations. The expected response was calculated by adding the individual responses of flies to vinegar and cVA. Only virgin females show a mixture synergism (**$P < 0.01$, *$P < 0.05$; Wilcoxon matched paired test). (*D*) Vinegar synergizes the cVA response in a ratio-dependent manner. Dose–response curve of cVA of PN responses ($\Delta F/F$) in glomerulus DA1 in virgin females with (striped line) or without vinegar (blue line) (10^{-2} concentration) in the background. A synergistic response is only visible at a 1:1 concentration of cVA and vinegar (**$P < 0.01$; Wilcoxon matched paired test).

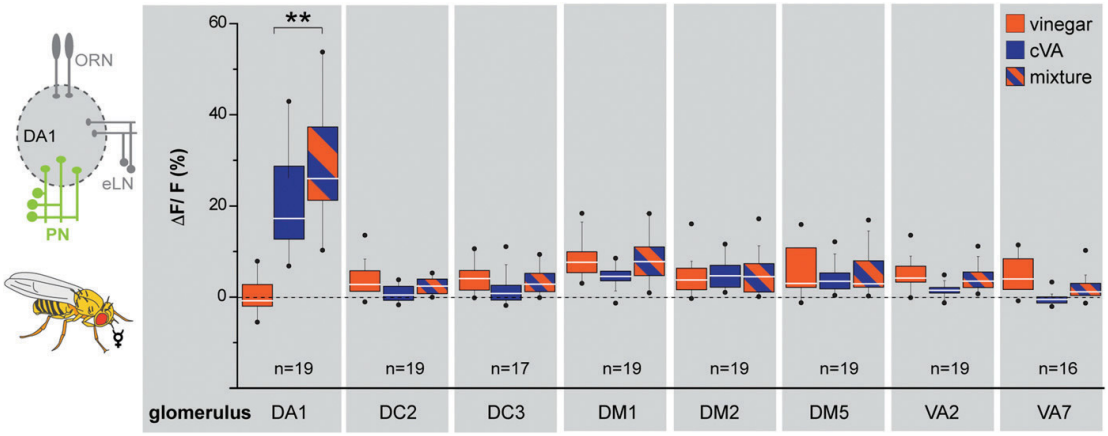

Fig. S2. Synergism in DA1 occurs in a glomerulus-selective manner. Box plots represent $\Delta F/F$ responses of PNs in different glomeruli in virgin females to vinegar (orange), cVA (blue), and their binary mixture (striped) (10^{-1} concentration). The white line in the box represents the median. Only glomerulus DA1 reveals a mixture synergism (**$P < 0.01$; Wilcoxon matched paired test).

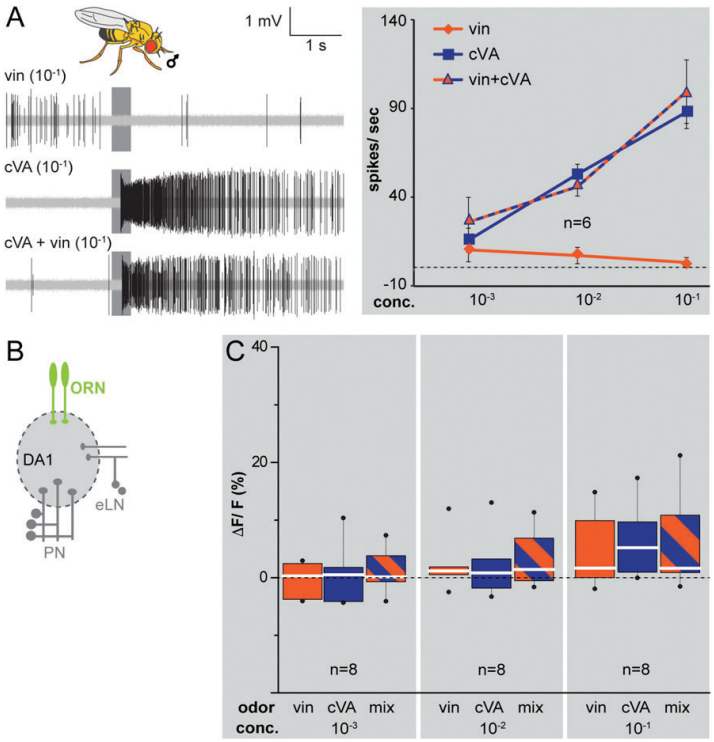

Fig. S3. Synergism does not occur at the sensory level in males. In vivo extracellular SSRs from the at1 sensillum expressing OR67d. (*A*, *Left*) Representative traces display the response of OR67d ORNs in virgin males to vinegar, cVA, and their binary mixture (10^{-1} concentration). (*Right*) Line curves represent the averaged responses (spikes per second) to vinegar (orange), cVA (blue), and their binary mixture (striped) at three different concentrations ($P > 0.05$; Wilcoxon matched paired test). (*B*) Schematic of the experimental approach: *UAS-GCaMP3* was expressed in the majority of ORNs (green) using *Orco-GAL4* in males. (*C*) Box plots represent $\Delta F/F$ responses of ORNs in glomerulus DA1 in males to vinegar (orange), cVA (blue), and their binary mixture (striped boxes). The white line in the box represents the median. The ORN response to the mixture is equal to the response to the stronger component (i.e., cVA) ($P > 0.05$; Wilcoxon matched paired test).

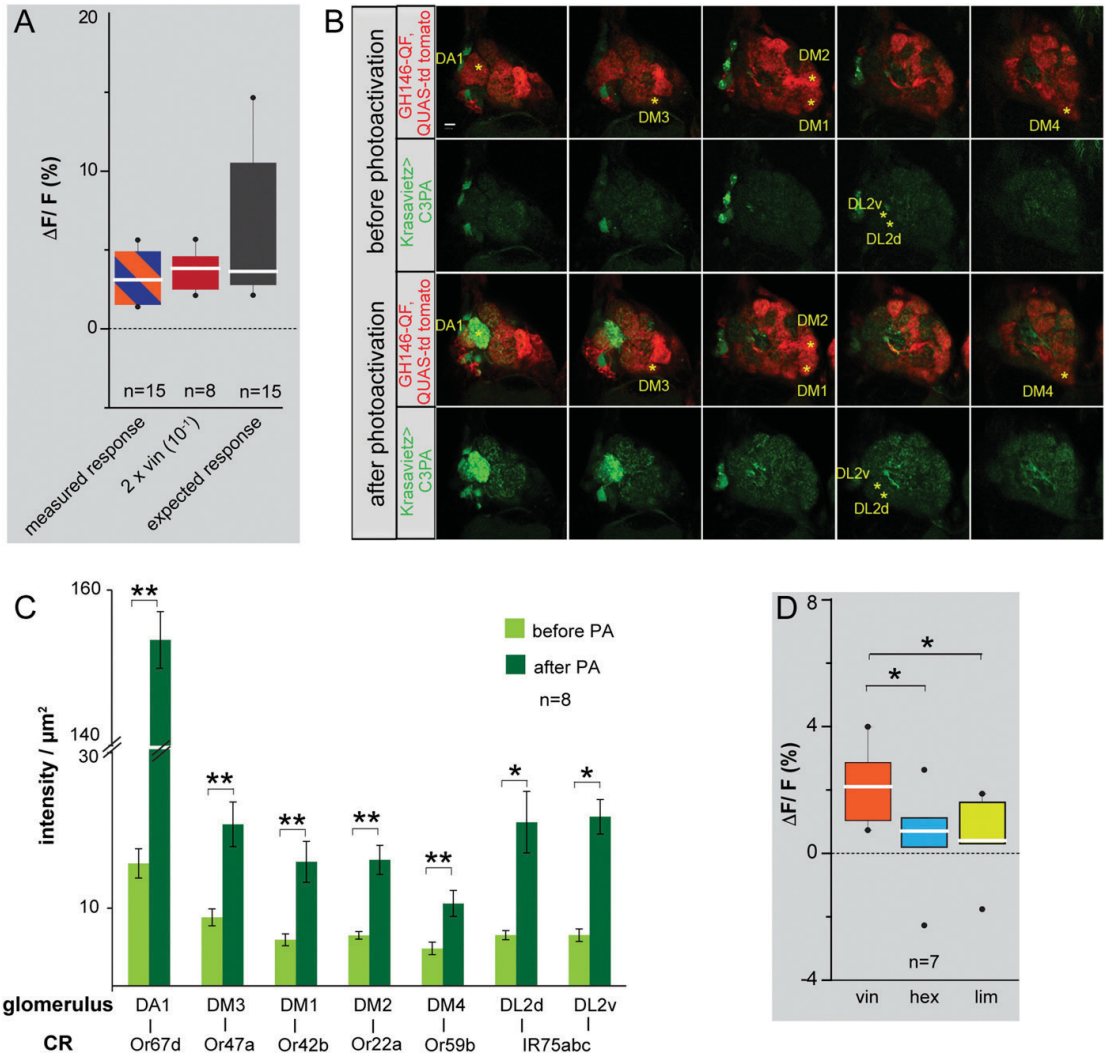

Fig. S4. Excitatory LNs do not show mixture synergism although they innervate both DA1 and vinegar-responsive glomeruli. (*A*) Comparison between expected (dark gray) and measured (striped) mixture response at 10^{-1} concentration and the response to double amount of vinegar at 10^{-1} of PNs in glomerulus DA1 in virgin females. The expected response was calculated by adding the individual responses of flies to vinegar and cVA. The measured mixture response is equal to the expected as well as the response to the double amount of the stronger component, (i.e., vinegar) ($P > 0.05$; Wilcoxon matched paired test). (*B*) Photoactivatable GFP *(UAS-C3PA)* expressed in eLNs using *Krasavietz-Gal4* (in green) and only the glomerulus DA1 was photoactivated. The different vinegar-responsive glomeruli (yellow asterisks) were identified based on their glomerular structure and visualized with *GH146 QF-QUAS td-tomato* in the background (in red). (*Upper*) The glomeruli at different focal planes before photoactivation. (*Lower*) The same glomeruli after photoactivation. (Scale bar, 10 µm.) (*C*) The fluorescence intensity per square micromete of GFP in different glomeruli (with their corresponding chemosensory receptor, CR) was quantified and compared before and after photoactivation. (**$P < 0.01$, *$P < 0.05$; Wilcoxon matched paired test). (*D*) Comparison of Krasavietz-positive eLNs response in DA1 to vinegar (vin), 1-hexanol (hex), and limonene (lim) at a concentration of 10^{-1}. The response to vinegar in DA1 is significantly higher than to the other two odors (*$P < 0.05$; Wilcoxon matched paired test).

DISCUSSION

General Discussion

This thesis elucidates the perception of pheromone and host volatiles in moths (*Heliothis virescens, Bombyx mori*) and flies (*Drosophila melanogaster*) mainly at three different levels of the olfactory pathway focusing on odor detection and the behavioral consequences. By this I investigated both the capability of the insect to detect specific volatiles and the subsequent behavioral consequences of these ecological relevant volatiles. My dissertation aims at providing a greater understanding of the larval pheromone detection system. Moreover, my investigations support evidence on sexual dimorphism and underline the importance of host detection in females. Finally, I highlight the complexity of chemical communication in both moths and flies by demonstrating that a host odor background can manifoldly influence the perception of pheromones dependent on the insect species, the odor presentation, the sex, and the mating status.

From molecules to detection

The larval perspective

Reproduction is one of the most essential tasks, which crucially relies on pheromone communication in insects. Lepidopteran males are attracted to the female-specific pheromone even over long distances emphasizing the highly sensitive pheromone system. In some species like *H. virescens*, female moths detect male-specific pheromone compounds that lead to an increase in the females' receptivity and supports her mate choice. Even in larvae a debate about the pheromone detection and its biological function has started recently (Poivet et al., 2012, He et al., 2010, Jin et al., 2015). Our study (chapter 1) supports the investigations obtained in *Spodoptera littoralis* (Poivet et al.2012) and *Bombyx mori* (He et al.2010): In all three species larvae have been demonstrated to detect female-specific pheromone components, whereby pheromone binding proteins (PBPs) may play an important role. Going a step further we could conclude that not only the sensory neuron membrane protein 1 (SNMP1) but also two adult pheromone receptors are involved in larvae pheromone detection. The adult pheromone receptors HR13 and HR6 are tuned to the two major pheromone components Z11-16:Ald and Z9-14:Ald. While these receptors are expressed in larvae, other pheromone receptors like HR14 and HR16, which respond to minor pheromone components (Baker2009, Wang et al.2011), seem to be not present. Indeed, larval OSNs of large basiconic sensilla (Laue2000) expressing HR13 and HR6 detect both above-mentioned pheromone components (chapter 1). In the adults the expression of some receptors depends on the sex, like HR13, which is only expressed in males but not in

females (Krieger et al.2004). In contrast, the expression of the two receptors HR6 and HR13 in larvae is independent of the sex supporting the importance of pheromone detection in larvae for both sexes. Furthermore, since all three molecular elements (HR, PBP, SNMP) are present in the larval pheromone detection system, the first chapter of my thesis strengthens recent assumptions that these proteins are involved in a sensitive pheromone detection. Together with previous findings (He et al.2010, Jin et al.2015, Poivet et al.2012) it can be considered that sharing the same proteins for the pheromone detection system with adults seems to be a specialty of the larvae of moths. In contrast to moths, *Drosophila* larvae do neither express the pheromone receptor Or67d nor other pheromone receptors (Fishilevich et al.2005, Kurtovic et al.2007) suggesting that *Drosophila* larvae do not respond to cVA or any of the known pheromone compounds. Notably, the dorsal organ of *D. melanogaster* larvae also seems to express SNMP1 (Fandino et al., in preparation), that is required in adult flies for a sensitive pheromone detection (Jin et al.2008) similar to moths. However, the function of this protein in larvae is so far unknown.

Since the molecular repertoire is similar in larvae moth and adult males, it might also be similar regarding their function: In adult males both components, Z11-16:Ald and Z9-14:Ald, together are sufficient to elicit pheromone attraction. The fact that the same two pheromone compounds are also detected during the larval stage suggests an ecological relevance also for the caterpillar. Moreover, the female-released pheromone might have a different role in larvae rather than serving as sex pheromone like in adult males, since the pheromone detection in larvae is independent on the sex. Findings in *S. littoralis* support such a hypothesis by demonstrating that larvae are more attracted to food containing the female pheromone than without the pheromone. Possibly, the advantage of sensing pheromones facilitates the detection of suitable feeding sites, since females release the pheromone while preferentially calling on host plants or they might release the pheromone during oviposition. Furthermore, foraging is experience-dependent in *Spodoptera* larvae (Carlsson et al.1999). Thus, the pheromone might support initial foraging on host plants. The behavioral consequence to detect female-released pheromone therefore needs to be investigated in larvae of *H. virescens*.

The females perspective

In adult moths and flies, trichoid sensilla detect pheromone compounds as demonstrated in one of the first electrophysiological studies on long-sized trichoid sensilla of male *B. mori* (Kaissling and Priesner1970, Kaissling et al.1978)(Boeckh et al.1965, Kaissling and Priesner1970, Kaissling et al.1978). While this applies for the male olfactory system, it becomes more complicated, when considering the female's detection system. Although females of several species are known to detect male-specific pheromone compounds with their trichoid sensilla, no single male-specific component could be found in the hair pencils of domesticated silkmoths *B. mori* (chapter 2). Nevertheless, these sex-specific structures were assumed to be involved in

olfaction rather than mechanosensation, since they are present in the first abdominal segment exclusively of males. One reason for the absence of male-specific hair pencil compounds in *B. mori* (chapter 2) could be the reduced amount of these volatiles in the investigated six days old males. The release of hair pencil compounds depends on the feeding during larval stage and seems not to be synthesized *de novo* (Bestmann et al.1993). Thus, the hair pencil volatiles were already weak in concentration or not present anymore when doing the odor collection. However, even in ten days old males of the coffee berry moth *Prophantis smaragdina* Lavogez et al.2017) were able to identify hair pencil compounds. Furthermore, in the closely related death's head hawkmoth *Acherontia atropos* the releases of hair pencil components was detectable even over several weeks (Bestmann et al.1993). Consequently, the observed lack of hair pencil compounds might be an effect of domestication, providing another hypothesis. A comparison of the volatile profiles obtained from hair pencil-like structures of *B. mori* with the one of its wild ancestor, *Bombyx mandarina*, might help to examine a putative domestication effect.

Going one step further in the second chapter, the characterization of two types of trichoid sensilla (medium- and long-sized) in female *B. mori* revealed that the female long trichoid sensillum (T1) is still the most puzzling sensillum type. In my studies, T1 neurons detected host volatiles, but no male-specific compounds, leading to the assumption that the sensillum serves as a detector for host cues instead of being a pheromone detector. Questioning this assumption, I still suggest that T1 sensilla of female *B. mori* detect pheromones because of the following reasons. First, there is only one functional type of long trichoid sensilla similar to those in males (Boeckh et al.1965, Kaissling and Priesner1970, Kaissling et al.1978, chapter 2) making up a total of 38% of all female sensilla (Steinbrecht1970). This suggests a specific function of this abundant type. Second, the results of my electrophysiological recordings further demonstrate that the male-specific compound acetophenone activates T1A neurons in *B. mori* weakly. However, the best ligand for T1A neurons is the host volatile linalool, acting as a male-specific hair pencil pheromone as well in other moth species (Bestmann et al.1993, Heath et al.1992). In general, hair pencil compounds in moths often derive from ingested plant material (Birch and Poppy1990) and are detected by the trichoid sensilla (Hillier et al.2006). It is therefore not surprising that female long trichoid sensilla detect plant-derived compounds like linalool, as it is conceivable that linalool or a structurally related compound act as a male-specific volatile in *B. mori*. Hereby, linalool activates long trichoid sensilla strongly as demonstrated in chapter 2 and might lead to acceptance of the male during courtship similar to other male-specific hair pencil volatiles (Hillier et al.2006). We only tested the behavioral consequence of mated female silkmoths towards linalool in a Y-maze (chapter 2). In order to test the hypothesis that linalool is a male-specific compound being involved in mate choice, virgins need to be tested and their behavior during courtship in the presence of linalool. Moreover, hair pencil compounds inhibit the attraction to female pheromones in conspecific males of several species (Lecomte et al.1998). For example, when adding hair pencil volatiles to the female-specific pheromone the upwind flight of a male is inhibited in *H. virescens* (Hillier et al.2007). Thus, continuing the hypothetical approach, linalool as putative male-specific compound might inhibit the attraction of males

towards bombykol in a similar way as in *H. virescens*. The interaction of linalool and bombykol has not yet been investigated at the behavioral level, but electrophysiological studies demonstrate that linalool inhibits the bombykol neuron (Kaissling et al.1989). This neuronal interaction suggests also an interruption of the pheromone response in male silkmoths and needs to be confirmed by behavioral investigations. A third reason for T1 sensilla of female *B. mori* to serve as a detector for pheromones is given by the fact that acids and benzaldehyde activate T1B neurons most strongly (chapter 2). Interestingly, benzoic acid and benzaldehyde are also present in hair pencils of some noctuid moth species (Birch and Poppy1990). However, the role of such compounds in *B. mori* is still unclear. In males the second neuron housed in long-sized trichoid sensilla is activated by bombykal, a putative minor sex pheromone component of conspecific females (Kaissling et al.1978). However, bombykal is also a sex pheromone component of many related moth species and antagonizes bombykol attraction in male *B. mori*. It is therefore considered that the bombykal-cell of male silkmoths ensures sexual isolation by preventing hybridization (Daimon et al.2012). In line with these assumptions, it might be possible that the second neuron of female T1 sensilla is involved in a comparable function by preventing interspecific mating. Acids or benzaldehyde might therefore be hair pencil compounds of closely related species rather than of male *B. mori*. Indeed, females show aversion towards isovaleric acid when tested behaviorally in the Y-maze (chapter 2). Whether virgin females also respond with aversion and how acids influence mate choice during courtship need to be further investigated.

In order to get an indication of the function for odor reception of the analyzed trichoid sensillum types I considered the reproduction status in the experiments. The reproduction status strongly affects the interaction of pheromones and host odors as demonstrated in moths (Barrozo et al.2010) and flies (chapter 3). The results of chapter 3 reveal that the synergistic mixture effect in *D. melanogaster* examined in virgin females is not present in mated females. As shown in several lepidopteran species, the reproduction status triggers also odor-evoked responses dependent on the context by reducing the sensitivity towards pheromones (Barrozo et al.2010, Gadenne et al.2001, Kromann et al.2015) and shifting the sensitivity towards host plant volatiles at the same time (Kromann et al.2015, Landolt1989, Masante-Roca et al.2007, Mechaber et al.2002). As a consequence, pheromone responses in males are inhibited after mating to prevent the male to mate with a conspecific female during the post-ejaculatory refractory period (Barrozo et al.2010, Gadenne et al.2001, Kromann et al.2015). Hereby, the reduced behavioral response towards the sex pheromone reflects the reduction of neuronal responses on the antenna of the male (Kromann et al.2015). However, the impact of the reproductive status on pheromone detection in female moths is so far unknown. In *Drosophila* we demonstrate that the neuronal response to cVA after mating is reduced in females (chapter 3) and that this leads to a suppression of the pheromone attraction (Lebreton et al.2015). Since females switch their behavior from finding a mating partner to oviposition after copulation, it is conceivable that also female moths experience a reduction in sensitivity to pheromones after mating, similar to male moths and female flies, and that the behavior matches physiological conditions. Accordingly, if

T1 is a detector for pheromone components like hair pencil volatiles (e.g. linalool or acids) in female *B. mori*, T1 neurons would be less sensitive towards these compounds after mating. However, the tuning properties of T1A neurons are only slightly affected by the reproductive status (chapter 2). A mating-dependent reduction in the sensitivity is observed after activating T1B neurons with isovaleric acid (chapter 2). Even though we demonstrated the strong activation of T1 neurons by certain compounds, the specific biological function of the ligands of T1 sensilla in female *B. mori* is still puzzling. Nevertheless, relevance in pheromone detection cannot be excluded. The presence of putative hair pencil compounds and the identification of acids or benzaldehyde in the habitat of silkmoths need further investigations.

Notably, a role of medium-sized trichoid sensilla (T2) as a detector for host volatiles can be presumed. In line with other studies demonstrating that trichoid sensilla detect plant compounds (Ghaninia et al.2014, Hillier et al.2006, Shields and Hildebrand2000), T2 neurons respond exclusively to host volatiles like cis-jasmone, methyl salicylate, (+)-linalool and α-terpineol (chapter 2). While mating decreases the sensitivity towards pheromone compounds (Barrozo et al.2010, Gadenne et al.2001, Kromann et al.2015), it simultaneously increases behavioral responses toward host volatiles (e.g. Masante-Roca et al.2007) and might therefore increase the sensitivity in host volatile-responsive neurons. Indeed, the sensitivity of OSNs, which are housed in T2 sensilla of female *B. mori*, is drastically increased after mating (chapter 2). Furthermore, T2 neurons respond most strongly to cis-jasmone, an important host cue for the larvae of *B. mori* (Tanaka et al.2009). Thus, T2 sensilla of female *B. mori* seem to have an important role in mediating host search by post-mating sensitization. The fact that OSNs of T2 sensilla of *B. mandarina* also respond most strongly to cis-jasmone (Figure 5A) underlines even more the ecological importance of T2 sensilla. Notable, the solvent used for the electrophysiological comparison of the two species *B. mori* and *B. mandarina* in Figure 5 was mineral oil. In contrast to the results in chapter 2, which demonstrated that cis-jasmone diluted in hexane was the best ligand of T2 neurons in *B. mori*, diluting odorants in mineral oil resulted in much stronger responses of T2 neurons evoked by linalool but not by cis-jasmone (Figure 5A, left panel). The lower release rate of mineral oil explains the differences, since it evaporates 20 times less cis-jasmone molecules than hexane does, while almost the same high emission rates of linalool were maintained for the two solvents (data not shown).

B. mori has lost capabilities due to domestication such as the ability to fly, whereas its wild ancestor *B. mandarina* lives in a natural habitat while the insect is still able to fly. It can be therefore considered that the detection of natural odors like host plants is more important for the survival of *B. mandarina* compared to the domesticated silkmoths *B. mori*. Furthermore it is conceivable that the lower selection pressure on *B. mori* led to a less sensitive system regarding host detection compared to its wild ancestor. Indeed, not only gustatory sensilla of *B. mandarina* respond much stronger to mulberry leaf extract than *B. mori* (Takai et al.2018), but also electrophysiological responses obtained from recordings of whole antennae upon stimulations to host volatiles are much stronger in *B. mandarina* (Bisch-Knaden et al.2014). These reduced

responses in *B. mori* might reflect a lower number of sensilla and might be an effect of domestication. In line with this, the receptive range of OSNs being housed in T2 sensilla seems to be different in wild and domesticated silkmoths (Figure 5A), which might also be an effect of domestication. The results of Figure 5A demonstrate that T2 neurons of *B. mandarina* show a tendency to be more narrowly tuned than T2 neurons of *B. mori*. Since *B. mori* is bread on artificial diet, OSNs might have lost the specificity to cis-jasmone resulting in a broader response spectrum. In contrast, the receptive range of OSNs being housed in long trichoid sensilla of *B. mandarina* is similar to that of *B. mori* and hence, not influenced by domestication (Figure 5B). The observation that only T2 sensilla seem to be influenced by domestication, but not T1 sensilla, indicates another biological role of the T1 sensillum type than solely mediating host search. Of course, a general conclusion can be drawn only after extending the initial investigations on the effect of domestication on the tuning properties of olfactory sensilla. In sum, I speculate that T1 sensilla are important for the detection of putative pheromones or silkmoths-related odors, while T2 sensilla are important in mediating host detection.

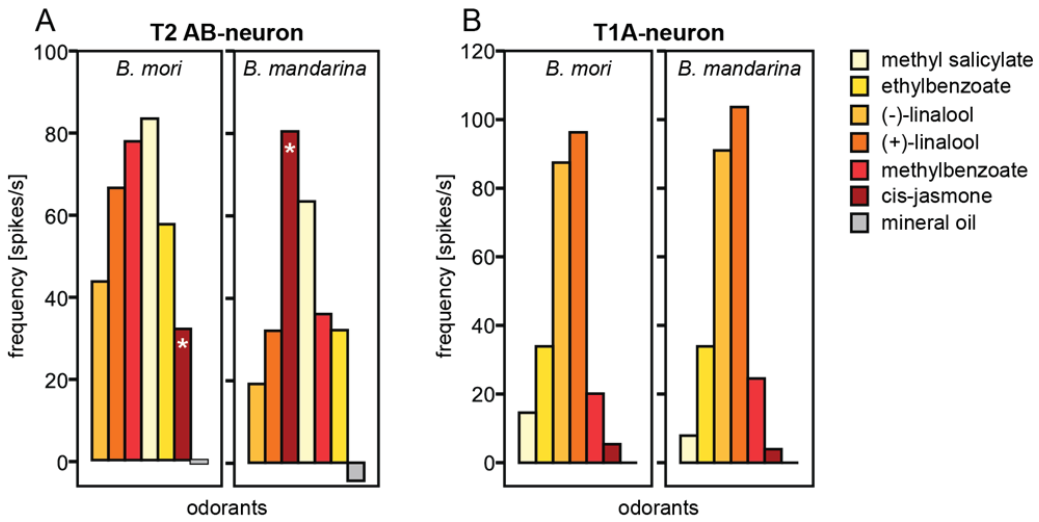

Figure 5: Receptive range of OSNs of trichoid sensilla in female silkmoths (E. Schuh unpublished). Bars represent the average maximum frequency (spikes/second) of OSNs, which are housed in T2 (A) and T1 (B) sensilla after odor stimulation (60 µg/µl in mineral oil) in domesticated *B. mori* and its wild ancestor *Bombyx mandarina*. Each color represents one of the odorants tested. **(A)** Receptive range of T2 neurons of *B. mandarina* is more narrowly tuned and respond most strongly to cis-jamone (dark red) compared to *B. mori*. Asterisks represent odor response to cis-jasmone. **(B)** Receptive range of T1 neurons is the same between domesticated and wild silkmoths.

From detection to behavior

Pheromone-host volatiles interaction in males and females

In nature, pheromones are never perceived alone as they are always embedded in a background of general odors. Insects have to deal with this situation of a rather complex olfactory environment. The field of volatile interactions in insects is highly controversial, because it has been demonstrated in various species that host volatiles either increase or reduce pheromone responses. According to these different findings, several explanations exist why environmental volatiles affect pheromone responses in various ways. Host volatiles might optimize orientation and prevent the insect from adaptation to an odor when perceiving alternating pheromone and host volatiles. Another possible explanation includes the release of sex pheromone components, on host plants in order to optimize mate finding and, by this, host volatiles might improve the perception of pheromones in males. The interaction of pheromone and plant volatiles has been studied intensively in the moth *H. virescens* (Hillier and Vickers2011, Pregitzer et al.2012, chapter 3). Here, certain plant volatiles reduce the responses to the major (Z11-16:Ald) and to the minor (Z9-14:Ald) sex pheromone component on the male antenna (Hillier and Vickers2011). Pregitzer and co-workers (2012) demonstrated that the suppression of Z11-16:Ald by plant volatiles occur at the level of the receptor HR13. Since both pheromone components are behaviorally relevant in male *H. virescens*, we therefore asked which behavioral consequence the pheromone-plant interaction might have and whether plant volatiles also reduce male pheromone attraction (chapter 3). Wind tunnel experiments demonstrated that the same plant volatiles with comparable concentrations as used for physiological studies also lead to a reduction in pheromone attraction in males. Nevertheless, GC-MS analysis of the headspace of host plants revealed that the tested concentrations of plant volatiles never occur under more natural conditions. Such high doses could also not be found in plants being damaged by larvae, although larval damage affects and increases volatile emission of plants (reviewed by Dicke and Loon2000). The absence of a change in behavior, when adding a host plant odor bouquet to the pheromone, implies first, that pheromone-plant interactions in *H. virescens* (Hillier and Vickers2011, Pregitzer et al.2012) rather represent an effect of supra-natural and artificial concentrations. Second, the natural host blend has a familiar composition (‘gestalt’) and therefore the male has no difficulties to follow the pheromone plume in a familiar background. In contrast, the occurrence of the single volatiles selected in the studies (Hillier and Vickers2011, Pregitzer et al.2012), chapter 3) impedes the pheromone detection in males due to confusion of the male since single volatiles are never perceived in nature. This confusion might finally lead to a reduction in the pheromone response of the male. To my knowledge, chapter 3 is one of the first studies, investigating the behavioral consequence of a suppression effect in pheromone-plant interaction. Only the group of T. Dekker (Hatano et al.2015) examined the reduced interaction of pheromones and host volatiles in a lepidopteran species at the behaviorally level. My results of chapters 3 comply with that of Hatano and co-workers (2015), who demonstrated that pheromone attraction of male *S. littoralis* is suppressed when adding a single herbivore-induced

plant compound. Our findings differ, however, when observing the interaction of the pheromone and a complete plant odor bouquet. While I could not observe a change in behavior when adding a host plant odor bouquet to the pheromone, in a two choice-wind tunnel assay Hatano et al. (2015) could show that *Spodoptera* males are significantly more attracted to an undamaged host plant compared to a damaged one in combination with the pheromone. These differing findings might be a result of methodical constrains, which I will discuss in the following. Different to the study of Hatano et al. (2015), males could not choose between two stimuli in chapter 3 of my thesis. Furthermore, in order to analyze odor-guided behavior I excluded the impact of visual cues by using the headspace of host plants instead of placing the plant itself in the wind tunnel as done by Hatano et al. (2015). Furthermore, Hatano et al. (2015) defined attraction as oriented upwind flight and thus it cannot be excluded that males directly contacted the pheromone source regardless on the physiological state of the host plant. I extended the analysis of odor-guided behavior by quantifying those moths that directly contacted the pheromone source. When investigating pheromone attraction of males in terms of sexual communication and mating using the wind tunnel, the source contact is the final stage of the flight as it better reflects mate finding (Cardé2016). In contrast, upwind flight without source contact implies the interruption of mate search and does not result in mate finding. In line with these considerations, the results of chapter 3 show that the suppression effect of pheromone and single plant volatiles is only present in males showing source contact, whereas their upwind flight toward the pheromone is not impaired. This result demonstrates that from the upwind flight of the moth we cannot necessarily deduce the number of moths finally contacting the source and, hence, conclude the attractiveness of the odor source. Moreover, I further extended the analysis of odor-guided behavior by dissecting the flight pattern into the number of source contacts, upwind speed, and the flight angle of the moth. We observed that none of the behaviors is affected by adding a headspace of host plants to the pheromone plume. Considering the results presented in this thesis along with the fact that most studies in lepidopteran investigated an increase in the pheromone attraction of males when adding host volatiles (Deng et al.2004, Gurba and Guerin2016, Light et al.1993, Reddy and Guerrero2000, Schmidt-Büsser et al.2009), I suggest that host volatiles do not diminish male pheromone attraction in nature.

The role of male-specific hair pencil pheromones in female mate acceptance and mate choice is the focus of several studies. *H. virescens* males also release such hair pencil compounds and influence the mate acceptance in females (Hillier et al.2006). Nevertheless, how host volatiles influence the female pheromone reception and the behaviorally consequences of this interaction is unknown and need to be addressed. In the fourth chapter of this dissertation we examined the interaction of a male-specific pheromone (cVA) and a complex food odor (vinegar) in *D. melanogaster*. This study represents the first investigation elucidating the mechanism behind the interaction of cVA and vinegar by examining several levels of the olfactory pathway from OSNs within the periphery to the AL up to the behavioral consequences. The function of cVA is complex and differs from the role of moths' pheromones as sole sex attractants. cVA is a multifunctional pheromone affecting the behavior of both males and females dependent on the

context (Ejima2015). The complexity and context-dependency of cVA is also reflected by its interaction with host volatiles as vinegar enhances cVA responses (synergism) specifically in virgin females (chapter 4). The receptivity of females during courtship is therefore enhanced by vinegar in virgin flies only.

In *Drosophila,* interactions between general odorants (Kundu et al.2016) or between CO_2 and host volatiles (Turner and Ray2009) are demonstrated to occur at the level of the OSNs. In contrast, SSR in at1 sensilla of *D. melanogaster* revealed no influence of vinegar on cVA-responsive neurons (chapter 4). Instead, the interaction was observed in projection neurons (PNs) in a glomerulus-specific manner. Vinegar is a complex blend that attracts vinegar flies (Becher et al.2010) by activating of a set of receptors (ORs\IRs) based on the composition of several components (Semmelhack and Wang2009). Since, the major compound of vinegar does not synergize cVA responses (chapter 4), a specific activation pattern evoked by vinegar might therefore be necessary to trigger this specific synergistic mixture interaction in PNs. The questions arose, first, whether the exact ratio of vinegar is necessary in this odor composition to induce this synergistic effect of vinegar and cVA and second, how modifications of the ratio of vinegar affect this interaction. It might be similar as the perception of sex pheromones, where variation of the female-released sex pheromone blend in moths causes a loss of pheromone attraction in males (Klun et al.1979, Ramaswamy and Roush1986, Vickers et al.1991). Does the modification of the vinegar blend lead to a loss of the specific olfactory information about the food source and hence result in a reduction of the synergistic mixture response? In order to answer these questions further investigations on the cVA-vinegar interaction are necessary by modifying the ratio of the vinegar components. If the interaction depends on a combinatorial activation of glomeruli within the AL by vinegar, I suggest examining another food odor such as a banana blend in order to investigate any enhancement of the cVA response. By studying the interaction of cVA together with other food blends, a general synergistic effect of food odors on cVA perception can be considered.

Both examples of pheromone-host volatile interaction, showed in chapter 3 and 4, demonstrate the complexity of pheromone communication in two different species: moths and flies. Chemical interactions depend on many factors, e.g. the chemical class and the concentration of odorants, the combination of the volatiles within a blend, the nutritional state of the insect or its sex. All these factors determine an interaction already within the same species as demonstrated in *Drosophila*. Accordingly, the food odorants phenyl acetic acid and phenyl acetaldehyde influence courtship exclusively in male flies (Grosjean et al.2011) and conversely, vinegar enhances female receptivity during courtship, without affecting the courting male (chapter 4). Flies feed, mate and oviposit on rotten fruits and in the presence of vinegar (Laturney and Billeter2014, Markow and O'Grady2008), hence, they always encounter host volatiles. It therefore appears logical that host volatiles, representing suitable oviposition sites and food resources for larvae, enhance the pheromone receptivity in females in order to optimally support one of the major tasks: reproduction and survival of the offspring. Although pheromone-plant

interaction does not occur in *H. virescens* (chapter 3), several examples demonstrate that enhanced mixture interactions of pheromones and host plants also occur in the male lepidopteran system (reviewed in Deisig et al.2014) underlining the complexity of pheromone interactions. Further studies are necessary to test the hypothesis that host plants influence the pheromone detection of female moths as well.

Complexity of nature

Why are some ORs more specific than others? In other words: why are some ORs narrowly tuned and are activated by a single volatile, whereas most ORs are broadly tuned and interact with several odor compounds? The pheromone system in male moths represents the best example of narrowly tuned and highly specialized PRs being expressed in OSNs of trichoid sensilla. Olfactory information are processed via a so-called labeled line principle (reviewed in Haverkamp et al.2018), meaning the detection of a pheromone compound immediately results in a characteristic pheromone-guided behavior, such as intense wing fanning and positive anemotaxis toward the pheromone source in *B. mori*. An example of a non-pheromonal compound is an olfactory cue in *D. melanogaster*, namely geosmin, making the fly to innately avoid harmful microbes (Stensmyr et al.2012). The general understanding of a labeled line is the detection of specific odorants of significant behavioral importance like pheromones. Females of *B. mori* also show odor-evoked behavior towards single volatiles (chapter 2): While the plant compound cis-jasmone elicits attraction and wing flapping behavior, (+)-linalool leads to an increase in oviposition. It is conceivable that single, highly ecological relevant volatiles can support the detection of host plants in females by indicating a suitable oviposition site and are, therefore, necessary to elicit a specific behavior. The findings of chapter 2 are in line with investigations obtained in females of *Manduca sexta* (Bisch-Knaden et al.2018). In the latter, females show a specific behavior, namely oviposition and attraction, upon stimulation with single odorants. In *Drosophila,* it has been shown that specific odorants of a food odor, e.g. limonene, trigger a specific behavior such as oviposition (Dweck et al.2013). Thus, in line with previous studies, the results of chapter 2 underline the importance of specific, ecologically relevant olfactory cues on the one hand. On the other hand, a comparison of the results obtained in chapter 2 with the behavioral performance of female silkmoths towards a natural blend is of high interest, since plant odors also often belong to a combinatorial system based on the ratios and the combination of specific components (e.g. Riffell et al.2009, Spaethe et al.2013a). By this, it can be examined whether the single relevant volatiles tested in chapter 2 are sufficient to trigger oviposition or attraction toward a host blend such as mulberry leaves.

The importance of a host blend is highlighted in chapter 4, which demonstrates that only a complex blend of vinegar and not acetic acid alone (major compound of vinegar), affects the interaction with the pheromone cVA. In another species I showed, however, that male

pheromone attraction is not affected by a background of a host plant (chapter 3) underlining the complexity of pheromone-host interaction in insects.

Systems have to be simplified in order to control experiments and to scientifically investigate a specific phenomenon. However, the more experiments reflect natural conditions the more the control over the experiments is lost, since the number of variables to be taken into account increases rapidly. These variables may be wind turbulences, changing environmental background odors, or the internal state of an insect, as well as factors such as parasitoids and predators, to name just a few. In chapter 3, I intended the setup to be as natural as possible. When investigating pheromone-plant interactions at the behavioral level I tried to mimic a female calling on a plant by releasing the corresponding volatiles out of a single nozzle (pulsed pheromone source) surrounded by circular arranged nozzles (continuous background plant volatiles). I was therefore able to mimic a more natural situation by extending previous laboratory investigations to get a better understanding of the ecological relevance.

Future prospects in moths and flies

In the present thesis I gained insights into the world of pheromones and host volatiles and its complexity at the sensory, processing and behavioral level in males and females. By investigating moths and flies, I used a variety of techniques in order to understand basic principles of pheromone and host volatile detection and to understand the more complex ecological relevance including volatile interactions. In addition, I highlight that we still have only a rudimental understanding of the complexity of the olfactory system. When investigating the detection of pheromone and host volatiles, one topic needs to be further studied and is touched only briefly in this thesis: sexual dimorphism. So far, studies in males mainly focused on pheromone detection while research in females put the emphasis mainly on host detection. My thesis revealed that some basic principles still remain elusive, as for example: Are volatiles, which are detected by long trichoid sensilla of females, similarly coded as pheromones in males based on the labeled line principle? Females often lack long trichoid sensilla and thus, the specific biological function of this sensillum type in females is of particular interest and still unclear (chapter 2). Activation of OSNs of T1 sensilla in female silkmoths immediately lead to a specific behavior as demonstrated in the second chapter for isovaleric acid and indole. These volatiles are detected by T1B neurons and cause aversive behavior. How are sex differences in the detection of host volatiles by medium-sized trichoid sensilla reflected in the behavior? Male moths often possess not as many medium trichoid sensilla as females (Steinbrecht1970) and these particular sensilla are often not characterized in males. An extension of the systematical analysis of the tuning and coding properties comparing males and females are important to understand the principles of odor detection and sexual dimorphism.

Genetic manipulations in other species than *Drosophila* have become possible very recently. They are therefore still limited in their extent and time consuming as well, because of longer generations times of moths, but they become important when investigating the odor processing in higher brain centers of moths, which is still not clearly understood. In male lepidopteran, the representation of the two subsystems of the AL processing either pheromones or host volatiles is maintained in the lateral horn (Zhao et al.2014). What are the coding principles for sexual dimorphism in higher brain centers? Is the sexual dimorphism of the AL also maintained? How does the representation of male- and female-specific pheromones and host volatiles look like in higher brain centers of female moths? Complicating the topic, in moths the odor ligands of different ORs are often unknown as well as which OR is expressed in which sensillum type. My thesis strengthens the fact that we cannot necessarily extrapolate from the detection and coding properties of the olfactory system in *Drosophila* to moths. As an example: *D. melanogaster* detects acids and amines mainly via IRs (Silbering et al.2011). In moths ORs might be strongly involved in the detection of such compounds as it can be observed in *M. sexta* (personal communication Richard Fandino). In this species an Orco knockout mutant reveals a drastic reduction of antennal responses to hexanoic acid suggesting that mainly ORs are involved in acid detection. In line with this finding for *Manduca*, OR45 and OR47 of *B. mori*, which are considered to be expressed in long trichoid sensilla of female silkmoths, seem to respond most strongly to acids and amines (Anderson et al.2009, chapter 2). New genome engineering tools, which have been developed in the past years, enable efficient gene knockout in *B. mori* and can therefore be used in order to study the function of single ORs in moths. By using such a method the receptor BmOR56, which is highly specific for the detection of cis-jasmone in *B. mori* larvae, should be studied in detail in adult silkmoths, since females are highly sensitive to cis-jasmone (chapter 2).

A general question arises when examining volatile interaction. Why do some interactions occur at different levels of the olfactory pathway either already in OSNs on the antenna or in the antennal lobe or even further in the brain? Does it enable a higher complexity in the olfactory system?

Finally, the most difficult part is the insect in its natural habitat as it requires further investigations for a better understanding of the ecological relevance of pheromones and host volatiles, their interaction and their behavioral consequences in males and females.

My dissertation contributes to a deeper understanding in the detection of pheromones and host volatiles by specific sensillum types on the antenna of male and female moths and flies. Furthermore, the results provide insights how this detection is translated into a specific odor-driven behavior by emphasizing the behavioral consequence. I elucidated how larvae are able to detect female pheromone components. Furthermore, by characterizing the detection properties of female *B. mori,* I highlighted the importance of host volatiles for female moths. Furthermore, we

showed evidence that volatile detection strongly depends on the reproduction status. Last, by investigating volatile interactions in two different species, I demonstrate the diversity of odor interactions. Moreover, my results suggest that some effects studied in the laboratory might be an effect of using unnatural conditions and would not occur in nature.

Chemical communication is not only biochemistry, because even equipped with modern techniques there is still something magical: Researchers will always be fascinated about the system, its sensitivity and how nature deals with the volatile situation.

SUMMARY

The environment of an insect contains a huge variety of volatiles affecting the decisions insects make in their life due to their sense of smell. Such an odor-driven behavior is dependent on many factors such as the chemical class of the odor molecules, the species, the sex, the life stage, the motivation or even the habitat, to name but a few. Odor molecules are detected by specialized hair-like structures on the antenna, so-called sensilla. Here the chemical information is first transformed into an electrical signal by the sensory neurons; subsequently the information is processed in the antennal lobe before being transferred to higher brain centers. Moths and flies are highly dependent on pheromones and host volatiles in order to find a mating partner or host plants for feeding and oviposition, respectively. Pheromones and host volatiles do not only differ in their function regarding chemical communication, but they are often also detected and processed in different subsystems of the olfactory system of moths and flies. However, both subsystems can influence each other which can have an impact on the insect behavior. In this dissertation I aimed at a deeper understanding of the complexity of chemical communication. Throughout my thesis I elucidated the role of pheromones and/or host volatiles in different life stages and sexes of moths and demonstrated the impact of specific host blends on pheromone-driven behavior in moths and flies including the involved mechanism.

In the first chapter we addressed the question if larvae of the budworm *Heliothis virescens* are able to detect female-specific pheromone and which molecular elements are involved in the pheromone detection. Using electrophysiological methods and immunolabeling we demonstrated that *Heliothis* larvae detect two important female-released sex pheromone components regardless of their sex, and that all molecular elements are expressed (HR6, HR13, SNMP1, PBP1, PBP2) in the larval antenna, as used in adult males for the detection of the same compounds. Thus, our results extent previous investigations on the larval pheromone system of other species and suggest that the molecular elements involved provide a highly sensitive pheromone detection system in larvae similar to adult males. Although the behavioral consequence of pheromone detection in larvae needs further investigation, it seems feasible that sex pheromones in larvae serve a different role than in adults.

In the second chapter I focused on the female point of view. Detection of pheromones in males and females is mediated by specialized sensilla, namely long trichoid sensilla. However, not all female moths are able to detect female- or male-specific sex pheromones. Female silkmoths do neither detect the female-specific pheromone bombykol nor bombykal, the second pheromone component of *B. mori*. In order to test whether OSNs being housed in trichoid sensilla of female *B. mori* are activated by male-specific compounds and host volatiles, I identified ecological relevant compounds by gas chromatography mass spectrometry of putative important odor sources. When performing single sensillum recordings using such ecological relevant volatiles I

characterized two types of trichoid sensilla (long- and medium-sized) and confirmed the detection of plant volatiles in these sensillum types. My results suggest a significant role of medium-sized trichoid sensilla in host detection since mating increased the sensitivity of neurons being housed in this sensillum type drastically. The biological function of long-sized trichoid sensilla is not yet fully understood. Moreover, in order to study the behavioral consequence of relevant odorants, which are detected by trichoid sensilla, I was able to establish a two-choice behavioral assay, the Y-maze, for female silkmoths. I observed several odor-driven behaviors such as attraction, aversion, wing flapping and oviposition. Although females of the domesticated moth *B. mori* are considered to be almost stationary throughout their life, I could show that females still show innate behaviors by the detection of ecological relevant compounds.

Independent of the volatiles the insect perceives, volatiles never occur alone in nature. Pheromones are always sensed in a background of various plant odors. In *Heliothis virescens* certain plant volatiles reduce neuronal pheromone responses on the antenna of males. In the third chapter, I therefore investigated the behavioral consequence of this interaction by studying the impact of single plant volatiles versus a complete host blend on pheromone-driven flight behavior in a wind tunnel. Although single volatiles reduced pheromone attraction in male *H. virescens*, a complete host blend did not affect the flight behavior and the navigational strategies of males. These results emphasize the importance of using ecological relevant stimuli in order to study odor-driven behavior in insects. In fact, male moths have no difficulties to find a calling female on a host plant.

Notably, in some species host blends seem to affect pheromone-guided behavior in males by enhancing the detection of pheromone compounds. Moreover, recent studies have shown that the food odor vinegar enhances pheromone attraction specifically in virgin females of *Drosophila melanogaster*. I therefore asked in my final chapter which neuronal mechanisms are involved in the pheromone-host interaction in female vinegar flies. The results of this chapter demonstrate an enhanced mixture interaction of the complex blend vinegar and the pheromone cVA in the cVA-responsive glomerulus of virgin females. In contrast to peripheral interactions observed in some moth species, the detection of cVA in trichoid sensilla is not affected by vinegar as we demonstrate in electrophysiological recordings. Using genetic manipulations it could be shown that this synergistic mixture effect derives in the antennal lobe network and is mediated by electrical synapses between excitatory local interneurons and projection neurons. After investigating the mechanism behind we asked the question, which behavioral consequence such a synergistic mixture interaction might have. Our results demonstrate that vinegar enhances the receptivity specifically of female flies during courtship underlining the advantage of mating in the presence of food in order to guarantee a feeding site for the female and its offspring. Altogether, the two last chapters highlight the diversity of pheromone host interaction in two different insect species.

With each of the four chapters of my thesis I was hoping to break another small piece of the ‘magic code’ of chemical communication. At the same time, my results open up new questions

in the field of pheromone and host perception in insects and highlight the diversity and complexity of the olfactory system.

ZUSAMMENFASSUNG

Die Umgebung eines Insekts enthält eine große Vielfalt an flüchtigen Substanzen. Diese tragen maßgeblich dazu bei, welche Entscheidungen Insekten aufgrund ihres Geruchssinns in ihrem Leben treffen. Ein solches geruchsgesteuertes Verhalten ist abhängig von vielen Faktoren wie zum Beispiel der chemischen Klasse der Geruchsmoleküle oder der Art, dem Geschlecht, dem Entwicklungsstadium, der Motivation oder sogar dem Lebensraum des Insekts. Geruchsmoleküle werden durch spezialisierte, haarähnliche Strukturen an der Antenne, sogenannte Sensillen, aufgenommen. Hier wird die chemische Information zuerst von den sensorischen Neuronen in ein elektrisches Signal umgewandelt. Anschließend werden die Informationen im Antennallobus verarbeitet, bevor sie an höhere Hirnzentren weitergeleitet werden. Motten und Fliegen nutzen Sexuallockstoffe (Pheromone) und Pflanzendüfte, um einen Paarungspartner bzw. Wirtspflanzen zur Nahrungsaufnahme und Eiablage zu finden. Pheromone und Wirtsdüfte unterscheiden sich nicht nur in ihrer Funktion als chemisches Kommunikationsmedium, sie werden oft auch durch verschiedene Subsysteme des olfaktorischen Systems von Motten und Fliegen detektiert und verarbeitet. Beide Teilsysteme können sich jedoch gegenseitig beeinflussen, was sich auf das Insektenverhalten auswirken kann. Mit meiner Dissertation habe ich ein tieferes Verständnis über die Komplexität chemischer Kommunikation angestrebt und die Funktion von Sexuallockstoffen und Wirtsdüften von verschiedenen Entwicklungsstadien und Geschlechtern von Motten aufgeklärt sowie den Einfluss von spezifischen Wirtsdüften auf Pheromon-getriebenes Verhalten bei Motten und Fliegen gezeigt - einschließlich des involvierten Mechanismus‘.

Im ersten Kapitel wurde untersucht, ob Larven der Baumwolleule *Heliothis virescens* das Weibchen-spezifische Pheromon detektieren können und welche molekularen Elemente dabei beteiligt sind. Mit elektrophysiologischen Methoden und Immunmarkierungen konnten wir zeigen, dass *Heliothis*-Larven unabhängig von ihrem Geschlecht zwei wichtige, von den Weibchen freigesetzte Komponenten des Sexuallockstoffes detektieren und dass, wie auch in adulten Männchen, alle molekularen Elemente (HR6, HR13, SNMP1, PBP1, PBP2) in der Larvenantenne exprimiert werden. Unsere Ergebnisse erweitern somit frühere Untersuchungen zum Pheromonsystem von Larven anderer Arten und legen nahe, dass die beteiligten molekularen Elemente in den Sensillen der Larven eine hochsensible Pheromondetektion ermöglichen, wie sie auch bei erwachsenen Männchen zur Pheromondetektion genutzt werden. Obwohl die Verhaltenskonsequenz, die der Pheromondetektion bei Larven zu Grunde liegt, weiter untersucht werden muss, scheint es möglich, dass Sexuallockstoffe in Larven eine andere Rolle spielen als bei Erwachsenen.

Im zweiten Kapitel lag mein Fokus auf dem Weiblichen Geruchssinn. Die Aufnahme von Pheromonen wird durch spezialisierte Sensillen, nämlich lange trichoide Sensillen, bei

Männchen und Weibchen vermittelt. Nicht alle weiblichen Motten sind jedoch in der Lage, weibchen- oder männchenspezifische Pheromone zu detektieren. Weibliche Seidenspinner riechen weder das weibchenspezifische Pheromon Bombykol noch Bombykal, die zweite Komponente des Sexuallockstoffes von *B. mori*. Um zu überprüfen, ob die Riechsinneszellen der trichoiden Sensillen in Weibchen von *B. mori* durch männchen-spezifische Düfte und Wirtsdüfte aktiviert werden, identifizierte ich mittels Gaschromatographie-Massenspektrometrie aus möglichen, relevanten Geruchsquellen ökologisch relevante Verbindungen. Die Ableitung einzelner Sensillen mit solchen ökologisch relevanten Düften ermöglichte mir die Charakterisierung zweier verschiedener trichoider Sensillentypen (lange und mittelgroße), welche Pflanzendüfte detektieren. Meine Ergebnisse deuten darauf hin, dass bei der Detektion von Wirtsdüften vor allem mittelgroße trichoide Sensillen eine wichtige Rolle für Weibchen spielen, da die Neuronen dieses Sensillen-Typs nach der Verpaarung der Weibchen drastisch sensitiver sind. Die biologische Funktion von langen trichoiden Sensillen ist noch nicht vollständig geklärt. Um die Konsequenzen relevanter Geruchsstoffe, die durch trichoide Sensillen detektiert werden, auf das Verhalten zu untersuchen, konnte ich außerdem einen Verhaltenstest, das Y-Maze, für weibliche Seidenraupen etablieren. Ich beobachtete verschiedene geruchsbedingte Verhaltensweisen wie Attraktion, Abneigung, intensiver Flügelschlag und Eiablage. Obwohl Weibchen der domestizierten Seidenspinner nahezu unbeweglich sind, konnte ich zeigen, dass sie durch die Detektion ökologisch relevanter Düfte immer noch angeborene Verhaltensweisen zeigen.

Unabhängig von der Art des Duftstoffes, der von Insekten wahrgenommen wird, kommen einzelne Duftstoffe niemals in der Natur vor. Pheromone werden immer gemeinsam mit den verschiedensten Pflanzendüften wahrgenommen. Pheromonantworten auf der männlichen Antenne von *Heliothis virescens* werden durch bestimmte Pflanzendüfte reduziert. Im dritten Kapitel untersuchte ich daher die Auswirkungen dieser Interaktion auf das Verhalten, indem ich den Einfluss einzelner flüchtiger Bestandteile einer Pflanze im Vergleich zu einer vollständigen Wirtsmischung auf Pheromon-gesteuertes Flugverhalten in einem Windkanal untersuchte. Obwohl einzelne Duftstoffe die Pheromonanziehung bei männlichen *H. virescens* reduzierten, beeinflusste eine vollständige Wirtsmischung das Flugverhalten und die Navigationsstrategien der Männchen nicht. Diese Ergebnisse unterstreichen die Wichtigkeit ökologisch relevanter Stimuli, um das geruchsgesteuerte Verhalten von Insekten zu untersuchen. In der Tat haben männliche Motten keine Schwierigkeiten ein Weibchen auf einer Wirtspflanze zu finden, welches ihren Sexuallockstoff abgibt.

Bemerkenswerterweise scheinen Wirtmischungen bei einigen Spezies das Pheromon-gesteuerte Verhalten bei Männchen zu beeinflussen, indem sie die Detektion von Sexuallockstoffen verstärken. Darüber hinaus haben neuere Studien gezeigt, dass der Futterduft Essig die Pheromongetriebene Anziehung speziell bei jungfräulichen Weibchen von *Drosophila melanogaster* verstärkt. Ich habe daher in meinem letzten Kapitel untersucht, welche neuronalen Mechanismen bei weiblichen Essigfliegen an der Pheromon-Futter-Interaktion beteiligt sind. Die

Ergebnisse dieses Kapitels zeigen, dass Jungfrauen eine synergistische Mischungsinteraktion durch den komplexen Essigduft und das Pheromon cVA in dem auf cVA-antwortenden Glomerulus aufweisen. Im Gegensatz zu peripheren Interaktionen, die bei einigen Mottenarten beobachtet wurden, wird die Aufnahme von cVA in trichoiden Sensillen nicht durch Essig beeinflusst, wie wir in elektrophysiologischen Untersuchungen nachweisen konnten. Mittels genetischer Manipulationen konnte gezeigt werden, dass dieser synergistische Mischungseffekt im Netzwerk des Antennallobens entsteht und durch elektrische Synapsen zwischen exzitatorischen lokalen Interneuronen und Projektionsneuronen vermittelt wird. Nachdem wir den zu Grunde liegenden Mechanismus untersucht hatten, untersuchten wir, welche verhaltensbedingte Konsequenz eine solche synergistische Mischungsinteraktion haben könnte. Unsere Ergebnisse zeigen, dass Essig die Aufnahmefähigkeit ausschließlich bei weiblichen Fliegen während der Balz fördert. Damit wird der Vorteil der Paarung in der Gegenwart von Nahrung unterstrichen, um eine Nahrungsquelle für das Weibchen und seine Nachkommen zu garantieren. Insgesamt heben die beiden letzten Kapitele die Vielfalt der Pheromon-Wirtspflanzen-Interaktion bei zwei verschiedenen Insektenarten hervor.

Mit jedem der vier Kapitel meiner Dissertation hoffe ich, einen weiteren kleinen Teil des "magischen Codes" der chemischen Kommunikation aufzudecken. Gleichzeitig werfen meine Ergebnisse neue Fragen im Bereich der Pheromon- und Wirtspflanzenwahrnehmung bei Insekten auf und verdeutlichen die Diversität und Komplexität des olfaktorischen Systems.

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DECLARATION OF INDEPENDENT ASSIGNMENT

I declare in accordance with the conferral of the degree of doctor from the School of Biology and Pharmacy of Friedrich-Schiller-University Jena that the submitted thesis was written only with the assistance and literature cited in the text.

People who assisted in experiments, data analysis and writing of the manuscripts are listed as co-authors of the respective manuscripts. I was not assisted by a consultant for doctorate theses.

The thesis has not been previously submitted whether to the Friedrich-Schiller-University, Jena or to any other university.

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Place, date Elisa Schuh

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PUBLICATION LIST

Professional Journals

2017 Das, S., Trona, F., Khallafa, M. A., **Schuh, E.**, Knaden, M., Hansson, B. S., and Sachse, S.: Electrical synapses mediate synergism between pheromone and food odors in *Drosophila melanogaster*. Proceedings of the National Academy of Sciences of the United States of America, 114(46), E9962-E9971. doi:10.1073/pnas.1712706114.

2016 **Badeke, E.**, Haverkamp, A., Hansson, B. S., Sachse, S.: A challenge for a male noctuid moth? Discerning the female sex pheromone against the background of plant volatiles. Frontiers in Physiology, 7: 143. doi:10.3389/fphys.2016.00143.

Haverkamp, A., Bing, J., **Badeke, E.**, Hansson, B. S., Knaden, M.: Innate olfactory preferences for flowers matching proboscis length ensure optimal energy gain in a hawkmoth. Nature Communications, 7: 11644. doi:10.1038/ncomms11644.

Zielonka, M., Gehrke, P., **Badeke, E.**, Sachse, S., Breer, H., Krieger, J.: Larval sensilla of the moth *Heliothis virescens* respond to sex pheromone components. Insect Molecular Biology, 25(5), 666-678. doi:10.1111/imb.12253.

Oral presentations

Sept. 2017 **Badeke E.**, Hansson B.S., Sachse S., Bisch-Knaden S. The sense of smell in female silkmoths. 15th European Symposium for Insect Taste and Olfaction (ESITO), Villasimius, IT

Feb. 2017 **Badeke E.**, Sachse S., Bisch-Knaden S. The ecological significance of trichoid sensilla in female silkmoths. 16th IMPRS Symposium, International Max Planck Research School, Dornburg, DE

Sept. 2014 **Badeke E.**, Pregitzer P., Hansson B.S., Krieger J., Sachse S. Reception and Coding of Pheromone Signals in Insects. 5th Annual Meeting of the SPP 1392 "Integrative Analysis of Olfaction", Delmenhorst, DE (invited)

Sept. 2014 **Badeke E.**, Hansson B.S., Krieger J., Sachse S. A challenging task for a male noctuid moth: scenting the conspecific female sex pheromone in the background of plant volatiles. European Chemoreception Research Organization (ECRO), Dijon, FR (invited)

June 2014 **Badeke E.**, Pregitzer P., Hansson B.S., Krieger J., Sachse S. A challenge for a male moth: Scenting the female sex pheromone in the background of plant volatiles. Insect Chemical Ecology (ICE 14), State College, US

Feb. 2014 **Badeke E.** Pheromone-guided flight behavior in *Heliothis virescens*. 13th IMPRS Symposium, International Max Planck Research School, Dornburg, DE

Sept. 2013 **Badeke E.**, Pregitzer P., Hansson B.S., Krieger J., Sachse S. Reception and coding of pheromone processing in moth. 13th European Symposium for Insect Taste and Olfaction (ESITO), Villasimius, IT

June 2013 **Badeke E., Pregitzer P.**, Hansson B.S., Krieger J., Sachse S. Reception and coding of pheromone signals in insects. 4th Annual Meeting of the SPP 1392 "Integrative Analysis of Olfaction", Jena, DE (invited)

June 2013 **Badeke E.**, Pregitzer P., Krieger J., Sachse S. Pheromone-plant interaction in *Heliothis virescens*. SPP Olfaction: 3rd PhD Meeting, Jena, DE

Poster presentations

March 2017 **Schuh E.**, Haverkamp A., Hansson B.S., Sachse S. A challenge for a male noctuid moth? Discerning the female sex pheromone against the background of plant volatiles. 12th Göttingen Meeting of the German Neuroscience Society, Göttingen, DE

Feb. 2015 **Badeke E.** Confusion by the background: Try to smell your sex partner! 14th IMPRS Symposium, International Max Planck Research School, Dornburg, DE

June 2014 **Badeke E.**, Pregitzer P., Hansson B.S., Krieger J., Sachse S. A challenge for a male moth: Scenting the female sex pheromone in the background of plant volatiles. Insect Chemical Ecology (ICE 14), State College, US, Jun 2014

May 2014 Haverkamp A., Bing J., Yon F., **Badeke E.**, Knaden M., Hansson B.S. Odour-guided foraging behaviour of the hawkmoth Manduca sexta. SAB Meeting 2014, MPI for Chemical Ecology, Jena, DE

May 2014 **Badeke E.**, Pregitzer P., Hansson B.S., Krieger J., Sachse S. A challenge for a male moth: Scenting the female sex pheromone in the background of plant volatiles. SAB Meeting 2014, MPI for Chemical Ecology, Jena, DE

Sept. 2013 **Badeke E**., Pregitzer P., Hansson B.S., Krieger J., Sachse S. Pheromone-plant interaction in *Heliothis virescens*. 1st International Workshop on Odor Spaces, Hannover, DE

Sept. 2013 **Badeke E.**, Pregitzer P., Hansson B.S., Krieger J., Sachse S. Pheromone-plant odor interaction in *Heliothis virescens*. ICE Symposium, MPI for Chemical Ecology, Jena, DE

April 2013 **Badeke E.**, Pheromone-plant interaction in *Heliothis virescens*. 12th IMPRS Symposium, International Max Planck Research School, Jena, DE